Studies on Steroid Hydroxylations in the Adrenal Cortex

by

Rohan H. Wickramasinghe, M.A. Cantab.

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Department of Biochemistry,
Medical School,
University of Edinburgh.

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Certificate of Originality

I certify that the work described in this thesis are the results of my own research and that the thesis has been composed by myself.

Rohan H. Wickramasinghe

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SUMMARY

Methods were developed for the preparation of large quantities of bovine adrenal cortex mitochondria. Adrenodoxin and adrenodoxin reductase, two components of the steroid hydroxylases found in these mitochondria, were isolated and examined. These two enzymes are responsible for the transfer of reducing equivalents from reduced nicotinamide-adenine dinucleotide phosphate (NADPH) to adrenal mitochondrial cytochrome P450. Some of the factors which may affect their functioning in the supply of electrons for corticosteroid hydroxylations were also investigated.

The amino acid composition of adrenodoxin reductase, a flavoprotein, was determined. Experiments showed that the protein had an isoelectric point near pH 8.9. The maximal rate of electron transfer to dichlorophenolindophenol (DCPIP) took place at pH 6.5. The coenzyme, flavin - adenine dinucleotide (FAD), was found to dissociate from the protein in the course of enzyme purification. This resulted in the enzyme losing its activity which was however readily regained on addition of FAD. Addition of excess FAD however resulted in inhibition of the activity of adrenodoxin reductase in reducing DCPIP and of steroid hydroxylation by unfractionated mitochondrial enzyme extracts. It is suggested that this is due to the excess FAD being reduced and autoxidised and thus diverting the flow of electrons. The reduction of FAD by NADPH in the presence of adrenodoxin reductase was demonstrated under anaerobic conditions.

Gel-filtration of adrenodoxin showed its molecular weight to be about 12,000. Electrofocussing of this protein resulted in its denaturation at pH 5.0. Investigations as to the possible existence of different species of bovine adrenodoxin did not yield any decisive

evidence. The amino acid composition of the enzyme closely resembled published figures except in the values for the glutamic and aspartic residues.

The reconstitution of the adrenal mitochondrial steroid hydroxylating enzyme system from purified components confirmed that high proportions of adrenodoxin relative to cytochrome P450 gave maximal *in vitro* hydroxylation rates. The rates of hydroxylation by these reconstituted systems were influenced by changes in ionic strength or the inclusion of bovine serum albumin in the assay medium.

Excess NADPH or large excesses of adrenodoxin inhibit the reduction of cytochrome c by mitochondrial cytochrome P450 reductase (adrenodoxin plus adrenodoxin reductase). Excess concentrations of NADPH inhibited deoxycorticosterone (DOC) 11β-hydroxylation while the Km values for NADPH for DOC 11β-hydroxylation and cholesterol side-chain cleavage to pregnenolone appear to be different.

The adrenal cortical NAD-kinase is located in the cytoplasm of the cell. The specific activity of the enzyme is comparable to that of the enzyme in other tissues and was not found to be affected by treatment with ACTH (*in vivo* or *in vitro*) or 3'-5' cyclic AMP (*in vitro*). The principal effect of oxidised or reduced glutathione on DOC 11β-hydroxylation by intact adrenocortical mitochondria was a marked inhibition.

Evidence has been obtained that adrenodoxin and adrenocortical mitochondrial cytochrome P450 can interact with phospholipids to form isooctane-soluble proteolipids. A molecule of adrenodoxin appears to complex with four molecules of phospholipid. The phospholipid complex formed with cytochrome P450 contains 26 μg phosphorus each as phosphatidylethanolamine and as lecithin per mg protein extracted

into the isooctane phase. The proteolipid formed by the haemprotein gives a spectral extinction maximum at 420 nm in isooctane solution following reduction with dithionite and treatment with carbon monoxide.

The interaction of adrenodoxin and adrenodoxin reductase has been observed spectrophotometrically. This interaction is affected by inorganic salts such as sodium chloride as is the interaction of adrenodoxin and cytochrome P450. The ionic strength also affects the reduction of cytochrome c by adrenodoxin plus adrenodoxin reductase and DOC 11β-hydroxylation by the mitochondrial steroid hydroxylase. The different effects of various monovalent anions and cations on DOC 11β-hydroxylation were found to be directly related to the ionic radius of each ion. Several dipolar ions known to affect the dielectric constant of aqueous solutions were tested and found to affect the rate of steroid hydroxylations by adrenocortical mitochondrial enzyme. It is possible that the unequal effects of various monovalent inorganic ions on DOC 11β-hydroxylation are related to the effects of their ionic radii on the known variation of the dielectric constant with distance from a monovalent ion.

The hypothesis is advanced that adrenodoxin, an iron-sulphur protein component of mitochondrial but not microsomal steroid hydroxylases, is a regulatory particle. Its function of electron transfer for mitochondrial corticosteroid hydroxylations is effected as a shuttle between adrenodoxin reductase and cytochrome P450. The rate-limiting nature of this step could be a reason for the increased steroid hydroxylation observed in <u>in vitro</u> enzyme assays on addition of purified adrenodoxin which is not linked by phospholipid bonds to a distinct functional enzyme unit. The shuttling movement or

oscillation of adrenodoxin is thought to be affected by the composition of the intramitochondrial contents. This could constitute a regulatory mechanism of the biosynthesis of mineralocorticoids and other corticosteroids.

(Key terms - Adrenal cortex; mitochondria; corticosteroid hydroxylations; flavoprotein; adrenodoxin; iron-sulphur protein; cytochrome P450; phospholipid; proteolipid; dielectric constant; ionic radius; mineralocorticoid; electron transfer; protein-protein interaction)

"------ La fixité du milieu intérieur est la condition de la vie libre,independante: ------ La fixité du milieu suppose un perfectionnement de l'organisme tel que les variations externes soient à chaque instant compensées et équilibrées.Bien loin,par conséquent,que l'animal élevé soit indifférent au monde extérieur,il est au contraire dans une étroite et savante relation avec lui,de telle façon que son équilibre résulte d'une continuelle et délicate compensation établie comme par la plus sensible des balances. ------"

Claude BERNARD

in "Sur les Phénomènes de la Vie"

Paris,1878.

CHAPTER I

INTRODUCTION

A. Steroids

Steroids are biological compounds derived from the perhydro-cyclo pentenophenanthrene nucleus (Fig. 1A).

Of the members of the class derived from this nucleus the most abundant are the sterols (certain solid alcohols), the bile acids, the adrenocortical hormones, the sex hormones, the cardiac glycosides, the sapogenins and certain alkaloids. The fundamental chemistry of most naturally occurring steroids has been settled and in many instances total chemical syntheses have been described. Fig. 1B shows a perspective view of the stereochemistry of the nucleus, which is seen as a fairly flat molecule with trans linkages. Figs. 1D and 1C show the perspective views of a 5α-steroid (rings A:B - trans) and a 5β-steroid (rings A:B - cis) respectively. Equatorial and axial substituents are marked e-, a- respectively. The steroid substrates and products referred to in the present work are shown in Figs. 3, 4 and 6 together with other steroids metabolised in the adrenal cortex.

Micro-organisms are known to effect a number of steroid transformations including some of cholesterol (Capek, Hanc and Tadra, 1966). In higher animals the major sterol is cholesterol, which is synthesised within the body (see Bloch, 1954; Popjak and Cornforth, 1960). Cholesterol is a substrate of mixed-function oxidases in the liver and in endocrine tissues. In the liver microsomes the initial attack appears to be the hydroxylation at the 7α-position of the nucleus. Further hydroxylations occur in hepatic microsomes to give rise to a variety of bile acids and neutral sterols (see Scholan, 1969). The utilisation of cholesterol to produce bile acids by the liver constitutes the quantitatively important pathway of its degradation.

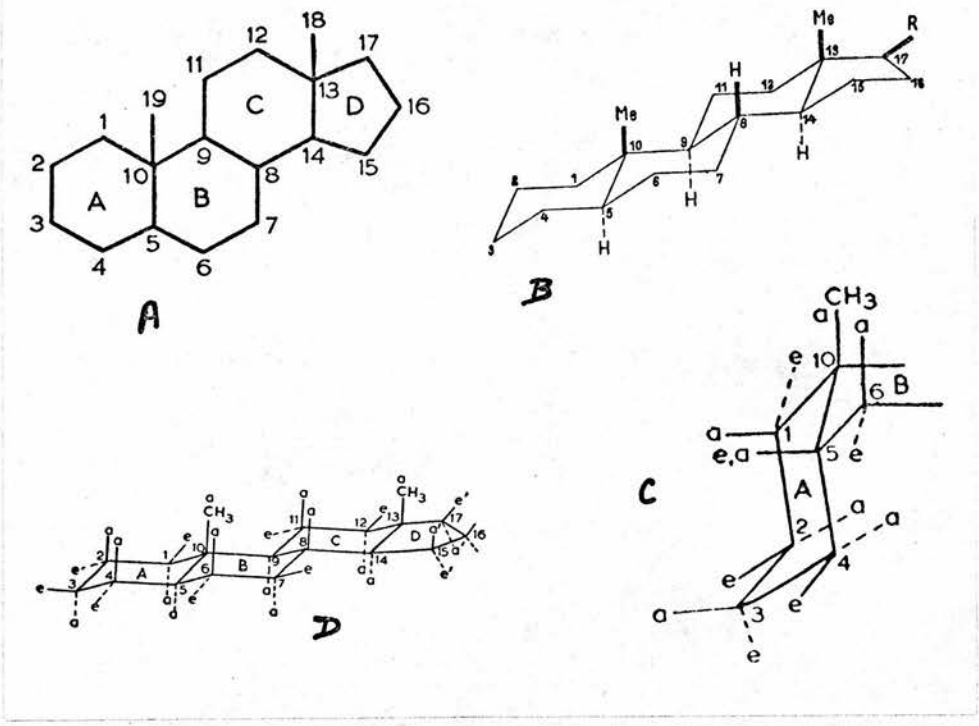


Fig. 1 Structure of steroids

A. Steroid nucleus
B. Perspective view of nucleus
C. 5 β-steroid (A:B-cis)
D. 5 α-steroid (A:B-trans)

(from Klyne, 1965)

Cholesterol is also utilised in the production of the steroid hormones. A considerable number of these steroids have been isolated and characterised and the relative proportions of each produced are known to vary according to the species and the endocrine tissue, and the physiological state of the latter and of the animal. Figs. 3, 4 and 61 show schemes of possible pathways of C21 steroid syntheses in the adrenal cortex. It is seen that the initial reaction leading to the biosynthesis of these steroids is the production of pregnenolone from cholesterol.

The C21 corticosteroids are usually loosely classified as mineralocorticoids or glucocorticoids. The mineralocorticoids such as aldosterone exert considerable regulatory effect on mineral metabolism while the glucocorticoids such as cortisol exert a controlling influence on "carbohydrate" metabolism. The difference in activities of the two classes is only relative but aldosterone, deoxycorticosterone and other mineralocorticoids provoke retention of Na^+, Cl^- and water but increase excretion of K^+ and nitrogen. They also affect the circulatory system in the cardiac and peripheral regions and the control of toxicity (Glaz and Vecsei, 1971). The glucocorticoids are known to increase the fasting blood-sugar and liver-glycogen concentration, increase diabetic hyperglycemia and glycosuria, stimulate gluconeogenesis from protein, increase protein catabolism and decrease glucose consumption. The androgens are C19 steroids formed both in the adrenal cortex and the interstitial tissue of the testis. Testosterone, androstenedione and dehydroepiandrosterone are members of this class, which is known to control, among others, aspects of sexual function, secondary sexual characteristics and urinary excretion of 17-ketosteroids. The oestrogens are C18 steroids and are produced mainly in the ovary and the placenta but also to a

lesser extent in the adrenal and testis. The biological effects and functions of each of these four classes not only overlap to some degree but they may also inhibit the production of each other by the negative feedback of ACTH production by the anterior pituitary or by the direct inhibition of the adrenals, probably by interference with protein synthesis. Glaz and Vecsei (1971) also discuss the steroids which are antagonists of the effects of other steroids (e.g. the antagonistic effects of progesterone and aldosterone).

B. Steroid-metabolising endocrine glands

The typical steroid hormones are produced mainly by the adrenal cortex, testis, ovary and placenta. As a broad generalisation the hormonal secretions of the adrenal cortex are concerned with regulation of the general chemistry and physiology of the individual, those of the gonads deal with sexual function and secondary sexual characteristics while the placental contributions are concerned with the maintenance of the reproductive system and foetoplacental unit during gestation. Sulimovici and Boyd (1969) review the evidence for the enzymatic transformations of cholesterol to pregnenolone and progesterone in the adrenal cortex, ovary, testis and placenta. A variety of other tissues also involved in the metabolism of these steroids include skin and vaginal mucosa (Frost et al, 1969), human endometrium and myometrium (Bryson and Sweat, 1969; Collins et al, 1969; Sweat and Bryson, 1969) and foetal tissue (Bloch, 1969) including the foetal adrenal.

(a) The Adrenal Gland

The adrenal gland of mammals is composed of medullary tissue, derived from the embryologic ectoderm, and the cortical tissue which has its origin in the mesoderm. It is highly vascularised, the minute

volume varying from 5.3 to 6.4 ml per gm of gland (Houssay and Molinelli, 1926). The adrenal cortex is composed of three layers - the zona glomerulosa, zona fasciculata and zona reticularis (innermost). Aldosterone biosynthesis is located predominantly in the zona glomerulosa (Simpson and Tait, 1955) while other hormones are however elaborated in the other two layers. The adrenal cortex has a very high lipid content which has been investigated by Chang and Sweeley (1963). They find that in the canine adrenal gland 90 per cent of the total adrenal lipids are neutral lipids, 90 per cent of the cholesterol exists in the esterified form and 60 per cent of the phosphorus is found in glycerylphosphorylethanolamine and glycerylphosphorylcholine. A recent detailed investigation of the rat adrenal cortex ultrastructure by Rhodin (1971) at magnifications of from 200x to 60,000x gives an excellent description of the histology of the gland and proposes a role for the lipid droplets in the elaboration and discharge of corticosteroid hormones. Depending on the zone being studied in a "normal" tissue a median cross section of a cell showed about 5 to 50 round to oval lipid droplets of varying size and electron density and about 50-75 mitochondria which varied greatly in size and ranged in shape from round and slightly oval to oblong and elongated.

(b) Adrenal mitochondria

The mitochondria of the cells of the adrenal cortex are largely similar to those found in other cells. Fig. 2 shows representations of mitochondrial structure with indications of the dimensions involved. The inset modified from Green and Perdue (1966) shows one diagrammatic interpretation of the mitochondrial substructure. In size the mitochondrion is about 3 μ (or exceptionally up to 10 μ) long and about

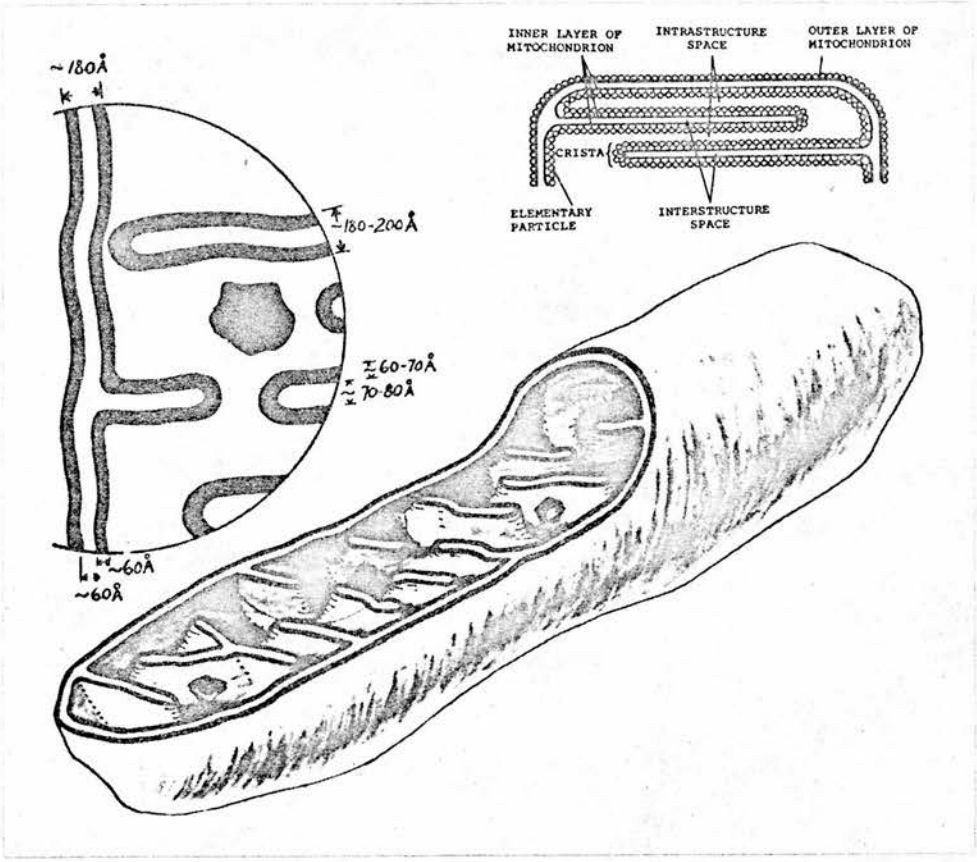


Fig. 2 Structure of a mitochondrion

(from Lehninger, 1960; original drawing of G. E. Palade)

Inset (upper right) modified from Green and Perdue (1966) shows a diagrammatic representation of the two membrane layers.

0.5 to 1.0 μ in width. *In vitro* the size is seen to be affected by the composition of the surrounding medium. Time-lapse cinematography at 60-second intervals has however shown the extraordinary variations in shape a single live mitochondrion can undergo (Frederic, 1958). As seen from Fig. 2 the mitochondria are composed of outer and inner membrane layers enclosing a semi-fluid matrix. The inner membrane is involuted to form the cristae which are usually transverse to the long axis, and are considered to be a development designed to increase the surface area of the membrane. The inner and outer membranes have a similar thickness (50-70 Å) and x-ray diffraction pattern but in other morphological, physical and chemical characteristics are very different (Ernster and Kuylenstierna, 1969). The methods of transfer of metabolites and other substances across these membranes are the subject of several theories, two of which are the transfer of charged ions under the influence of a membrane potential (Liberman and Skulachev, 1970) and the transfer of ions across the inner mitochondrial membrane by cytochrome c (Margoliash, Barlow and Byers, 1970). The passage of substances across these membranes and state of swelling of the organelle is governed by the physiological state of the mitochondrion and the composition of the fluid bathing it. It is however generally accepted that reduced nicotinamide nucleotides do not readily penetrate intact mitochondrial membranes. This and several other lines of evidence have resulted in the intramitochondrial localisation of the numerous mitochondrial enzymes in the matrix, inner membrane or outer membrane (see Ernster and Kuylenstierna, 1969). Thus Satre, Vignais and Idelman (1969), Yago and Ichii (1969) and Yago *et al.* (1970) identified cytochrome P450, DOC 11 β-hydroxylase activity and cholesterol side-chain cleavage activity in the inner

membranes of adrenal cortex mitochondria although Billiar et al. (1971) claim that the side-chain cleavage activity is located in the outer membrane.

C. Steroid hydroxylations in the adrenal cortex

Fig. 3 shows that some steroid hydroxylations take place in the mitochondria and some in the microsomes of endocrine tissues. The principal mitochondrial hydroxylations are 11β-, and 18-hydroxylations (Nakamura, Otsuka and Tamaoki, 1966; Omura et al, 1966) and cholesterol C20 and C22 hydroxylations leading to side-chain cleavage (Sulimovici and Boyd, 1969) while 17- and 21-hydroxylases are found in the microsomes. The conversion of pregnenolone to progesterone by a 3β-ol dehydrogenase and a Δ^5-3-ketosteroid isomerase is commonly believed to take place in the endoplasmic reticulum (microsomes) but recent reports show the existence of mitochondrial enzymes as well (Koide and Torres, 1965; Sulimovici and Boyd, 1968a).* The preferred cofactor for this dehydrogenase is NAD^+.

The hydroxylation of steroids in endocrine tissue (and in the liver) requires molecular oxygen and a supply of reducing equivalents from the cofactor NADPH (Boyd, 1970). Fig. 5 shows some pathways leading to the supply of reducing equivalents for corticosteroid hydroxylations. The role of cytochrome P450 in the mechanism of oxygen insertion into the steroid molecule in a postulated reaction sequence is shown in Fig. 7. The cholesterol side-chain cleavage reaction is atypical of steroid hydroxylations however in that although it possesses all the characteristics of a mixed-function oxidase the overall effect achieved is the result of several successive transformations in the course of which the steroid appears to remain firmly bound to the enzyme. The usual lack of success in

*Although Fig.5 shows the microsomal conversion of pregnenolone to progesterone, observations made during the present work support the possible presence of mitochondrial enzymes for these reactions.

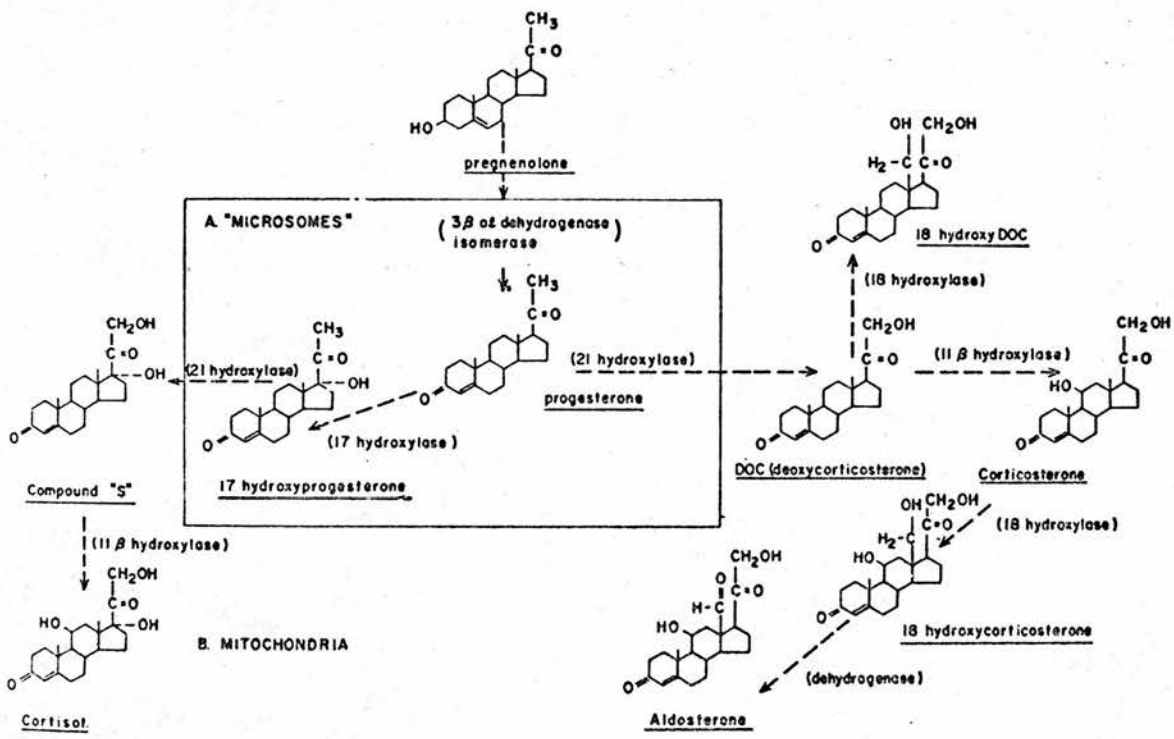


Fig. 3 Pathways of corticosteroid hydroxylations

(from Bransome, 1968)

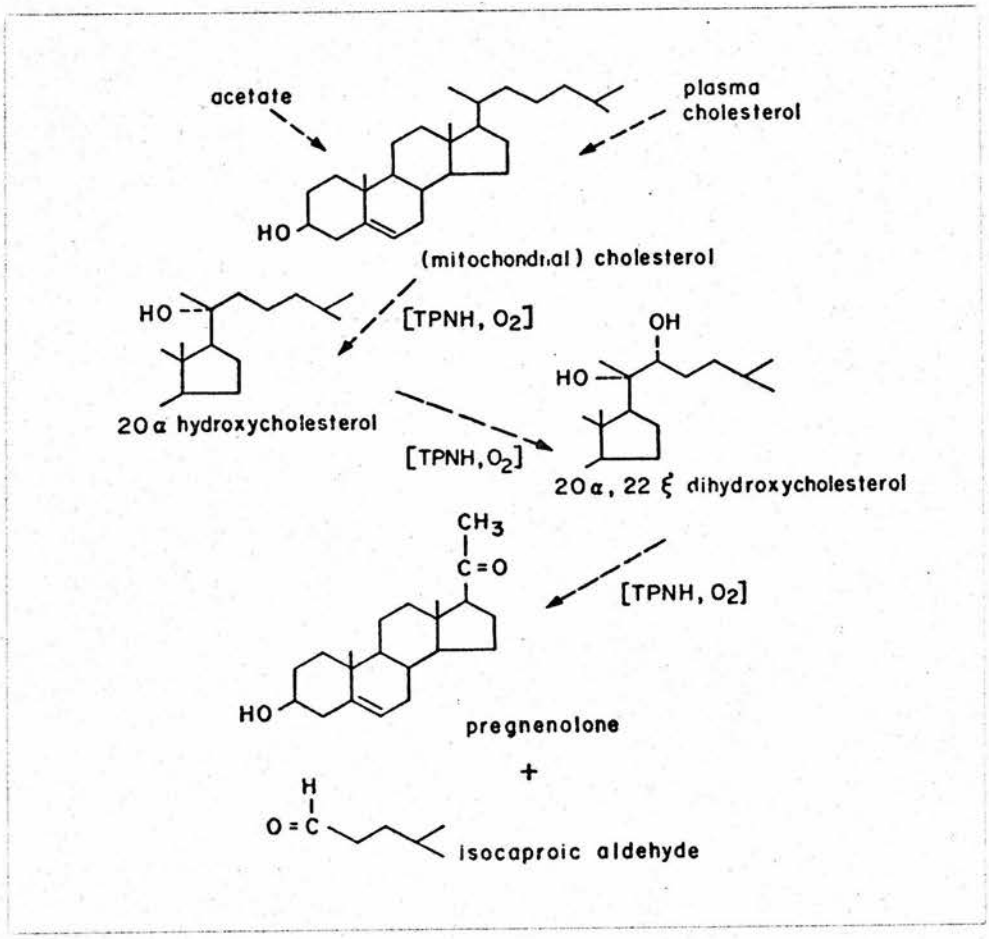


Fig. 4 A postulated pathway of biosynthesis of pregnenolone from cholesterol ("cholesterol side-chain cleavage")

(from Bransome, 1968)

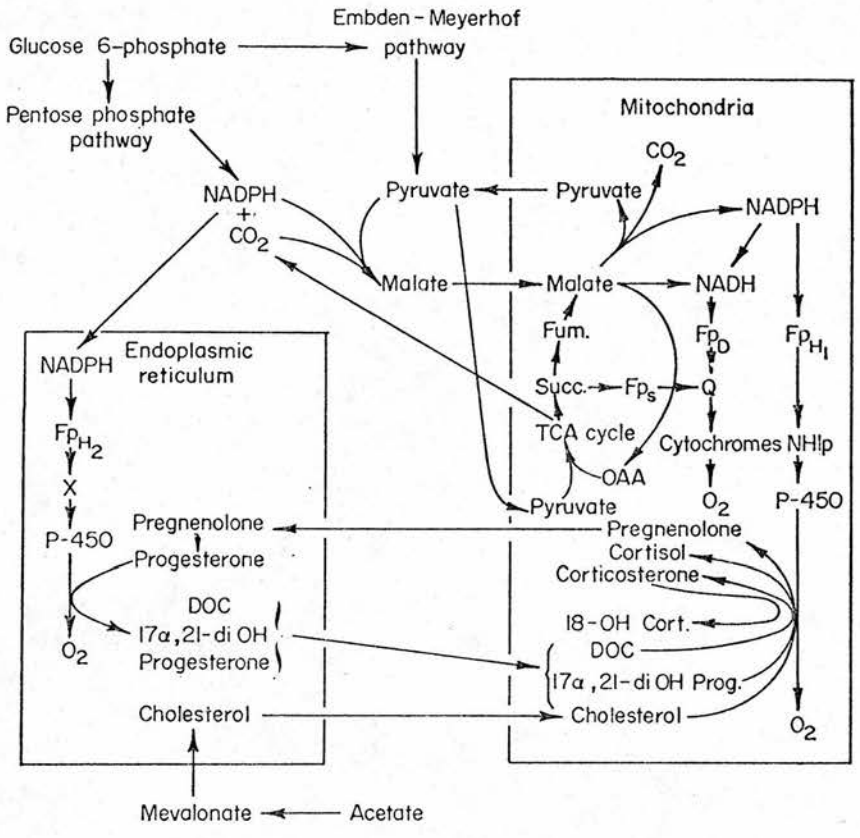


Fig. 5 Some metabolic activities known to occur in an adrenal cortical cell

(from Simpson, Cooper and Estabrook, 1969)

identifying intermediates between cholesterol and pregnenolone has hampered the elucidation of the reaction sequence but current ideas favour the scheme shown in Fig. 4.

Sulimovici and Boyd (1969) review earlier evidence for the above sequence as well as that in support of a minor pathway bypassing pregnenolone and leading directly to dehydroepiandrosterone and 2-methyl heptan-6-one. Burstein et al.(1970); Burstein and Gut (1969); Burstein, Kimball and Gut (1970) and Burstein, Peron and Williamson (1969) present fresh evidence on the major pathway while van Lier and Smith (1970a,b,c) report findings on cholesterol 20α-hydroperoxide as an intermediate in the conversion of cholesterol to pregnenolone by adrenal cortex mitochondria.

Bransome (1968) and Glaz and Vecsei (1971) discuss other steroid transformations in the adrenal cortex. Bransome (1968) and Sulimovici and Boyd (1969) also review evidence for the probable existence of different "pools" of unesterified cholesterol of which some are preferentially utilised for hormone elaboration and others are less accessible. Moses et al.(1969) have used autoradiography in an endeavour to localise adrenal cholesterol by electron-microscopy. It has also been shown in vivo and in vitro that cholesterol sulphate can be metabolised to the corresponding esters of pregnenolone and dehydroepiandrosterone without the removal of the sulphate radical (Raggatt and Whitehouse, 1966). From another approach Takemoto et al. (1968) have investigated the oxygen requirement of cholesterol side-chain cleavage while Hall (1967) reported that hyperbaric oxygen is inhibitory in this reaction.

D. Biological Hydroxylations

The requirement of molecular oxygen for steroid transformations

is not a universal characteristic of all biological hydroxylations. Another source of oxygen is the oxygen atom of a water molecule which is known to serve in the formation of 6-hydroxynicotinic acid from nicotinic acid, barbituric acid from uracil and in the 6-hydroxylation of the pteridine ring. The direct transfer of a hydroxyl group from one compound to another has been postulated from studies on the dehydroxylation of kynurenic and xanthurenic acids.

The term dioxygenases was employed by Hayaishi for the enzymes which introduce both atoms of an oxygen molecule into the substrate to form dihydroxy compounds such as that catalysing the formation of catechol from anthranilate. These enzymes also operate in the biosynthesis of the prostaglandins. The monooxygenases incorporate one atom of a molecule of oxygen into the substrate while reducing the other to water. The latter enzymes have been separated into the internal monooxygenases, such as lysine monooxygenase, which utilise reducing equivalents drawn from the substrate itself, and the external monooxygenases which require an external electron donor such as ascorbic acid (e.g. dopamine β-hydroxylase) or a reduced pyridine nucleotide. The three reactions (Hayaishi, 1969) may be designated as follows:

(a) Dioxygenation $\quad \text{Substrate} + O_2 \longrightarrow \text{Substrate}\begin{matrix}\diagup OH \\ \diagdown OH\end{matrix}$

(b) Internal monooxygenation

$$\text{Substrate} + O_2 \longrightarrow \text{Substrate} - OH + H_2O$$

(c) External monooxygenation

$$\text{Substrate} + O_2 + 2e^- \longrightarrow \text{Substrate} - OH + H_2O$$

The third type which includes the hydroxylation of camphor (Katagiri, Ganguli and Gunsalus, 1968), imidazoleacetate and phenylalanine (Hayaishi and Nozaki, 1969), drugs (Gillette et al, 1969),

pesticides (Casida, 1970), steroids and a variety of natural products is that usually described in referring to enzymic "mixed-function oxidation".

Mixed-function oxidases are complex enzyme systems on which intensive research effort has been directed over the last ten years. Both reduced nicotinamide-adenine dinucleotide-(NADH-) and reduced nicotinamide-adenine dinucleotide phosphate-(NADPH-) dependent hydroxylations have been found and a flavin group is often present in the enzyme system. In view of the diversity of locations and variety of substrates attacked extensive generalisation is impracticable but it may be noted that these enzyme systems are usually multicomponent and often act in a highly lipid environment due to the hydrophobic nature of many of the substrates.

The multicomponent nature of these systems means that at least one of the constituents of the chain is usually limiting the rate of hydroxylation under otherwise optimal conditions of factors such as substrate availability. The fact that some systems such as the hepatic microsomal hydroxylase are bound onto the cell membranes also make the study of their physiological functioning more difficult. It is however well established that both the hydroxylating activity and the cytochrome P450 and cytochrome P450 reductase contents of some mixed-function oxidases are highly inducible by substrates and other compounds (see Gillette et al, 1969), though not always to parallel extents. This high degree of inducibility of hepatic hydroxylases is of considerable significance in drug metabolism, since an administered drug may be broken down at different rates in different ~~hepatic~~ states of hepatic induction for example. Remmer (1969) states moreover that the induction of these microsomal enzymes is a completely non-specific phenomenon produced by a great variety of lipid-soluble compounds and indeed

McLean (1968) and others have investigated the possible effects of ingestion of coffee in causing small changes in drug metabolising activity. An additional property which influences the effects of mixed-function oxidases is that of "synergism". Casida (1970) describes the use of synergists to potentiate the effect of small quantities of pesticides. One of the effects of synergists in these systems may be to lessen pesticide hydroxylation (detoxification) at the locus of cytochrome P450, one of the enzymes of this system.

The composition of the mixed-function oxidase complexes is variable. Whereas a flavoprotein is usually the first enzyme of the sequence to accept electrons from the reduced pyridine nucleotide, from one to three other proteins have been postulated to be present in the different hydroxylating systems. Fig. 6 shows recent proposals of the enzymes involved in hepatic drug and pesticide hydroxylations (Casida, 1970). It is seen that two flavoproteins, cytochrome b5 and another haemprotein, termed cytochrome P450 (Omura and Sato, 1964a,b) are believed to participate. The requirement of cytochrome P450 has been demonstrated in many cases while the postulated involvement of cytochrome b5 in hepatic microsomal hydroxylations is still to be unequivocally established.

The involvement of another type of iron-protein, though this time of the electron-transferring non-haem iron variety, has also been demonstrated in some hydroxylating systems. This is seen in Table I which describes some differences between certain steroid hydroxylases. As regards steroid hydroxylations in the mitochondria of the adrenal cortex the work of Nakamura, Otsuka and Tamaoki (1966) and Omura et al.(1966) established the requirement of a flavoprotein, an iron-sulphur protein and cytochrome P450 for the transfer of reducing equivalents from NADPH to the site of hydroxylation (Fig. 7).

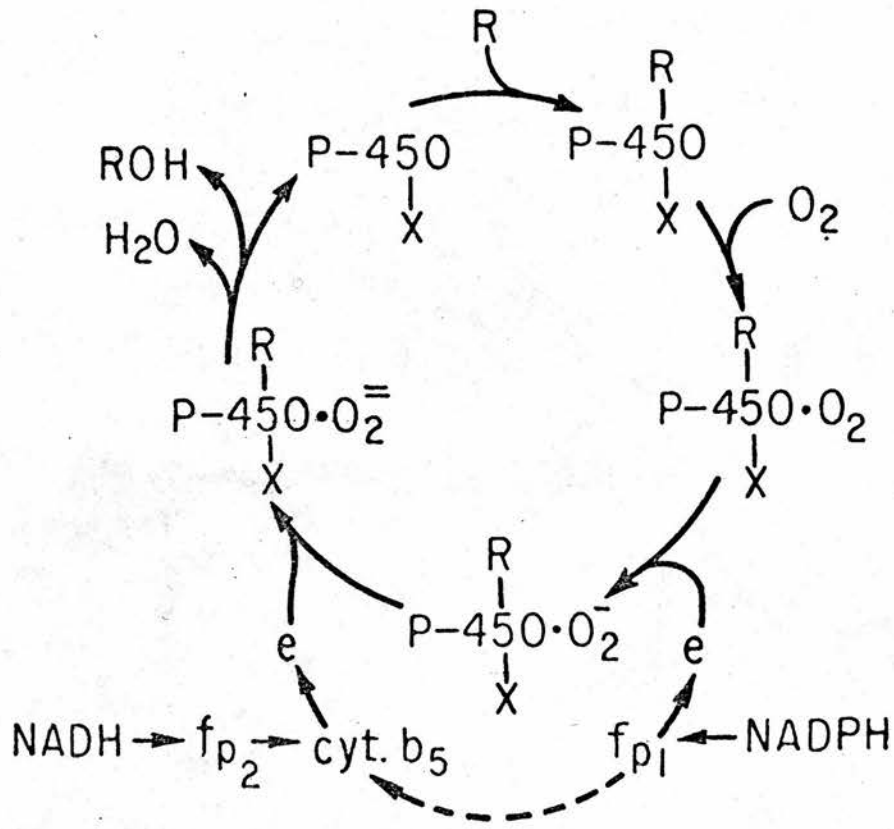


Fig. 6 Postulated mechanism of hepatic drug and pesticide hydroxylations

(from Casida, 1970)

Table I Comparison of some characteristics of three types of steroid hydroxylations

	Class A	Class B	Class C
1) Location -	non-endocrine tissue e.g. liver	endocrine tissue e.g. adrenal cortex	endocrine tissue
2) Location in cell -	microsomal	mitochondrial	microsomal
3) Type of product -	non-hormonal e.g. bile acids	hormonal and concerned with metabolic regulation (these hydroxylations on the major pathway of mineralocorticoid production)	hormonal
4) In vitro assay -	not inhibited by high ionic concentrations	highly susceptible to raised ionic levels	-
5) Iron-sulphur protein -	not identified in tissue and not involved in reaction e.g. 7α-hydroxylation	present and required for all hydroxylations viz. for cholesterol side-chain cleavage and 11β- and 18-hydroxylations	not identified and not required in enzyme system e.g. 21-hydroxylation

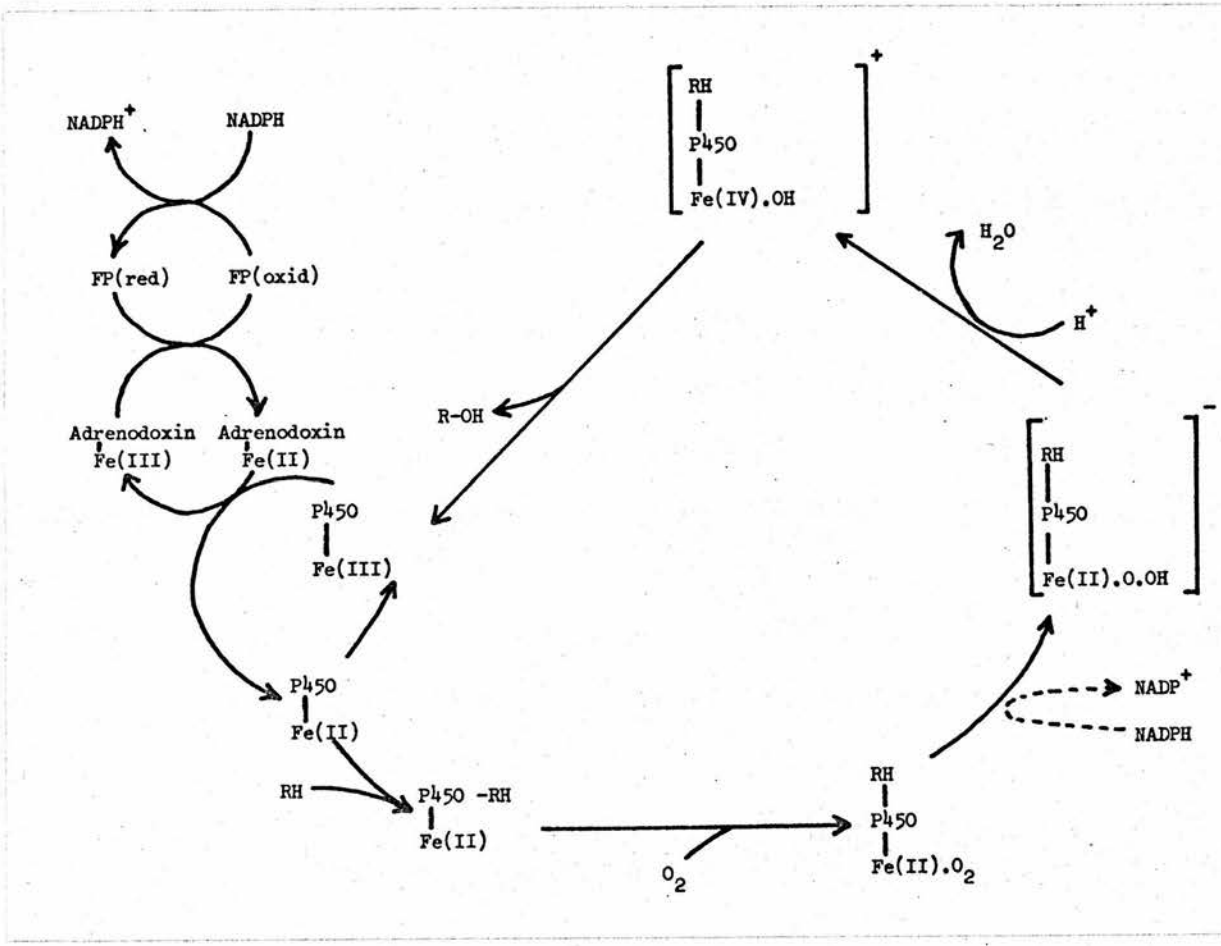


Fig. 7 A possible mechanism of steroid hydroxylation in the adrenal cortex mitochondrion

(adapted from Sih, 1969)

Considerable subsequent work as well as that in the course of the present work has confirmed the participation of these proteins in cholesterol side-chain cleavage and 11 β-hydroxylation in the adrenal cortex. Accounts are therefore presented below of the characteristics of the enzyme classes (and those of some examples) to which each of these proteins belong. The description of the iron-sulphur proteins is treated in greater detail with reference to adrenodoxin, the adrenal mitochondrial iron-sulphur protein.

E. Flavoproteins

Flavoproteins are those proteins containing and functionally dependent on flavin mononucleotide (FMN) or flavin-adenine dinucleotide (FAD). While the "nicotinamide-containing dehydrogenases" usually have a freely dissociable coenzyme, the flavin to protein-binding is generally more stable. These enzymes function as electron-transferring agents which accounts for the dependence on the flavin moiety. The catalysis by these enzymes depends on the cyclic acceptance by the flavin of one electron to give the half-reduced form, the semiquinone, and of two electrons to give the fully reduced form and subsequent reoxidation. The formation and functioning of oxidised, semiquinone and (or) fully reduced forms in flavoprotein catalysis have been investigated for several enzymes (see Slater, 1966) and lead to complexities in interpretation where the proteins contain several flavin groups. Certain flavoproteins are also known to contain both FMN and FAD (Trudgill, Du Bus and Gunsalus, 1966a).

While flavoproteins function generally in electron transport, the substrates or donors and acceptors of electrons are varied. The flavoprotein enzymes include several amino acid oxidases, glutathione reductase, phytoflavin, flavodoxin, xanthine oxidase, aldehyde oxidase, dihydroorotic dehydrogenase, NADH peroxidase, succinic

dehydrogenase, cytochrome b_2 (aerobic yeast L(+)-lactate dehydrogenase), ferredoxin reductase and components of electron-transport chains of various systems including mixed-function oxidases. Some of these enzymes such as xanthine oxidase and cytochrome b_2 contain iron in non-haem or haem forms while other enzymes such as xanthine oxidase and aldehyde oxidase also contain molybdenum. Flavoproteins containing non-haem iron are also known to have acid-labile sulphur and on removal of the flavin content present an extinction spectrum very similar to spinach ferredoxin (see Neims and Hellerman, 1970; Slater, 1966).

The FAD-containing monooxygenases include imidazoleacetate monooxygenase (Maki et al., 1966), p-hydroxybenzoate hydroxylase (Hosokawa and Stanier, 1966) and salicylate hydroxylase (Katagiri et al., 1965). Some of these enzymes have been crystallised. The bacterial lactonization of camphor is a mixed-function oxidation by a multienzyme system which includes an NADH-dehydrogenase (Trudgill, Du Bus and Gunsalus, 1966a,b).

Other flavoproteins of interest related to the present work are the ferredoxin reductases which have been isolated from a number of sources. Shin and Arnon (1965) showed that spinach ferredoxin reductase has a Km for NAD of 3.75×10^{-3}M while the Km for NADP is 9.78×10^{-6}M. In this instance NAD and NADP compete for the same site on the protein. The formation of a complex between ferredoxin and ferredoxin reductase has been extensively investigated by Foust and Massey (1967); Foust, Mayhew and Massey (1969)and Nelson and Neumann (1968,1969a,b). The transhydrogenase properties of ferredoxin reductase are stimulated by ferredoxin (Fredricks and Gehl, 1971; Nelson and Neumann, 1969a). Phytoflavin (Smillie, 1965) and flavodoxin (Mayhew, Foust and Massey, 1969; Mayhew and Massey, 1969) are two iron-free, FMN-containing

proteins which can apparently substitute enzymatically for ferredoxin in its functions with ferredoxin reductase in reactions such as the photoreduction of NADP by chloroplasts. Flavodoxin is synthesised as a "ferredoxin-substitute" when bacteria are grown in media deficient in iron.

The redox properties of flavodoxin (Mayhew, Foust and Massey, 1969) and a number of other flavoproteins (e.g. Estabrook, 1966) have been reported. The NADPH reduction of some flavoproteins (e.g. Estabrook, 1966) has been reported to occur on the millisecond time scale while reoxidation of others are known to take place with a half-time of about 150 m seconds (Erecinska and Storey, 1970). Since reoxidation of many reduced or semireduced flavoproteins takes place on exposure to air, reductive titrations are often carried out under anaerobic conditions (Masters et al., 1965). The investigations of Masters et al.(1965, 1971) and Ichikawa and Yamano (1969) were carried out on hepatic microsomal NADPH-cytochrome c reductase. The reduction of cytochrome c has been employed in the assay of some flavoproteins and with the ferredoxin/ferredoxin reductase system but is not thought to be the physiological activity of the hepatic microsomal enzyme. Hepatic microsomal NADPH-cytochrome c reductase is considered rather to be the enzyme which carries out NADPH-cytochrome P450 reduction in vivo. A similar flavoprotein transfers reducing equivalents from NADPH to cytochrome P450 in adrenocortical microsomes but reduction of adrenocortical mitochondrial P450 by NADPH (Fig. 7) requires the insertion of an iron-sulphur protein called adrenodoxin into the chain between the flavoprotein and the haemprotein (Estabrook, 1966; Kimura and Suzuki, 1967; Omura et al., 1966). Adrenodoxin is similar in many of its characteristics to the ferredoxins and other electron-transferring proteins containing non-haem iron and labile sulphur.

F. Iron-sulphur proteins

Iron-containing proteins may be divided into the haemproteins and the non-haem proteins (see also Table II). The latter class may be subdivided into the iron-storage and transporting proteins such as ferritin and transferrin, the oxygen-transporting haemerythrin and the electron-transferring non-haem iron proteins. These include those proteins which have variously been called "non-haem iron-", "iron-sulphur" and "ferredoxin-type" proteins. None of these terms is adequate but the first two are probably the most often used today for the whole class.

Historical background of research on "ferredoxins"

San Pietro and Lang (1958) were the first to isolate from green plants an enzymic factor which was required to enable spinach chloroplasts to carry out reduction of pyridine nucleotides. They termed this factor PPNR or "Photosynthetic Pyridine Nucleotide Reductase". What is now recognised to be the same enzyme was independently found to be needed for a variety of other reactions such as methaemoglobin- and haem-reduction. The identity of the enzymes required for the reactions was not appreciated till very much later and dates from around 1960 (Davenport, 1960; Hill and San Pietro, 1963; Valentine et al, 1963).

The name "ferredoxin" soon gained acceptance to denote a non-haem iron protein which mediated the electron-transfer required to carry out the various functions previously ascribed to the activities of different enzymes. The name was coined by D. C. Wharton and first applied to a protein containing non-haem iron and acid-labile sulphide which was isolated from Clostridium pasteurianum by Mortenson, Valentine and Carnahan (1962). A similar protein containing non-haem iron and acid-labile sulphide and participating in electron-

Table II Estimated distribution of iron[a] in Man

Compound	Percentage of body iron
Haemoglobin	70.5
Myoglobin	3.2
Storage Fe: ferritin and haemosiderin	26.0
Transport Fe: transferrin-Fe complex	0.1
Parenchyma or tissue Fe:	
Cytochrome c	0.1
Catalase	0.1
Other cytochromes, Fe-flavoproteins, adrenodoxin etc.	?

a) The total body iron is estimated to be 3.85 g in a 70 kg man.

(modified from Moore and Dubach, 1962)

transfer was independently isolated from Chlorella in Warburg's laboratory by Gewitz and Voelker (1962).

Following the isolation of ferredoxins from a variety of organisms and enzyme preparations catalysing a diversity of basic biological processes a remarkable burst of scientific activity has taken place in this field over the last decade. The efforts of many laboratories to clarify the structure and function of the "ferredoxins" has resulted in revealing the diversity of structure of these proteins in addition to the already recognised multiplicity of biological functions. The discovery of the rubredoxins furthermore demonstrated the existence of "ferredoxins" which do not possess acid-labile sulphide (or sulphur which is released as hydrogen sulphide on acidification).

Distribution

Certain non-typical "electron-transferring non-haem iron proteins" such as the non-haem iron components of the respiratory chain (see Bois and Estabrook, 1969), protein B_2 of ribonucleotide reductase (Brown et al., 1968) and cysteamine oxygenase (Rotilio et al., 1970) are omitted from the present account. Selected references of work on "typical" iron-sulphur proteins (ISP^S) of various types and sources are given below.

A. Ferredoxins (bacterial)

1. Clostridia (various species) - Hong and Rabinowitz (1967,1970a,b,c); Jeng, Devanathan and Mortenson (1969); Nitz et al.(1969); Sobel and Lovenberg (1966); Thauer et al.(1969).
2. Micrococcus - Tsunoda, Yasunobu and Whiteley (1968); Whiteley and McCormick (1963).
3. Butyribacterium - Valentine and Wolfe (1963).
4. Desulfovibrio - Buchanan (1969); LeGall and Dragoni (1966).

5. Methanobacillus - Buchanan, Lovenberg and Rabinowitz (1963); Buchanan and Rabinowitz (1964).

6. Peptostreptococcus - Gillard et al.(1965, 1966); Mayhew and Peel (1966).

7. Rhodospirillum - Losada, Whatley and Arnon (1961).

8. Soya bean nodule Rhizobium - Klucas, Koch and Evans (1968); Koch et al.(1970).

9. Chloropseudomonas - Telfer, Cammack and Evans (1970).

10. Rhodopseudomonas - Yamanaka and Kamen (1965).

B. Ferredoxins (plant and other)

1. Spinach ferredoxin - Marlborough, Hall and Cammack (1969); Matsubara (1968).

2. Parsley, pea leaves, alfalfa, taro, cotton, Leucena and other higher plants - Bendall, Gregory and Hill (1963); Katoh and Takamiya (1963); Mathieu, Miginiac-Maslow and Remy (1970); Rao (1969).

3. Green alga - Scenedesmus - Powls, Wong and Bishop (1969); Sugeno and Matsubara (1968).

4. Anacystis, Anabaena and Tolypothrix - Black, Fewson and Gibbs (1963); Smillie (1965); Tagawa and Arnon (1962).

5. Nostoc - Mitsui and Arnon in Arnon (1965b).

6. Phormidium - Keresztes-Nagy, Perini and Margoliash (1969).

7. Chromatium - Buchanan and Evans (1969).

8. Chlamydomonas - Mitsui (1970).

9. Chlorella - Gewitz and Voelker (1962).

10. Chlorobium - Buchanan, Matsubara and Evans (1969); Weaver, Tinker and Valentine (1965).

11. Euglena - Kimura and Ohno (1968).

C. Rubredoxins

1. Clostridia - Lovenberg and Williams (1969); Stadtman (1965).

2. Desulfovibrio - Laishley, Travis and Peck (1969); Newman and Postgate (1968).

3. Micrococcus - Bachmeyer, Yasunobu and Whiteley (1967, 1968).

4. Peptostreptococcus - Bachmeyer et al.(1968b).

5. Pseudomonas - Ichihara, Kusunose and Kusunose (1970); Kusunose et al.(1968).

D. High Potential Iron Proteins

1. Chromatium - Bartsch (1963); Evans, Hall and Johnson (1970).

2. Rhodopseudomonas - Dus et al.(1967).

E. Other iron-sulphur proteins

1. Putidaredoxin - Cooke et al.(1968); Moleski et al. (1970).

2. Beefheart mitochondrial ISP - Coleman, Rieske and Wharton (1964); Rieske (1965).

3. Azotobacter ISP^{s} - Shethna, Wilson and Beinert (1966).

4. Paramagnetic proteins (Cl. pasteurianum, A. vinelandii) - DerVartanian, Shethna and Beinert (1969).

5. Ferredoxin-type protein from Euglena - Johnson et al. (1968).

6. Molybdoferredoxin - Burns, Holsten and Hardy (1970); Mortenson, Morris and Jeng (1967).

7. Azoferredoxin - Bulen and LeComte (1966); Kelly (1969).

8. Succinic dehydrogenase ISP - King (1964); Zeylemaker, DerVartanian and Veeger (1965).

9. Dihydroorotic dehydrogenase (Zymobacterium oroticum) - Aleman et al.(1965).

10. Xanthine oxidase - Gibson and Bray (1968); Hart and Bray (1967).

11. Aldehyde oxidase - Aleman et al.(1965).

12. Hydrogenase - Nakos and Mortenson (1971a).

13. "Synthetic" ISP^S - McCarthy and Lovenberg (1968); Suzuki and Kimura (1967).

In addition to the above enzymes, iron-sulphur proteins which participate in the hydroxylation of steroids (see Fig. 7) have been isolated from the following organs -

1. Adrenal cortex (Adrenodoxin) - Kimura (1968); Omura et al (1966).
2. Testis (Testodoxin) - Kimura and Ohno (1968); Ohno, Suzuki and Kimura (1967).
3. Ovary - Ohno, Suzuki and Kimura (1967); Sulimovici (1968).
4. Placenta - Billiar and Little (1969); Mason (1970).

From the selected references given above on work done on each protein discovered may be obtained an idea both of the widespread occurrence of these proteins and of the considerable effort made over the past ten years into characterising them.

Functions and enzymatic assay techniques

No specific biological functions can as yet be ascribed to some of these proteins such as most rubredoxins (see DerVartanian, Shethna and Beinert, 1969; Dus et al, 1967). The others undergo alternate reduction and oxidation to effect electron-transport in several biological systems (see Figs. 7, 8 and 9). Reduction of these proteins may be effected by light or a variety of common reducing agents either with or without a ferredoxin-reducing flavoprotein as an intermediary.

The reduction of ferredoxin by solar light energy is used by plants to produce chemical energy (Eisenstein and Wang, 1969; Hall and Evans, 1969). The process is linked to NADP-reduction, photophosphorylation and oxygen absorption and evolution (Mathieu, Miginiac-Maslow and Remy, 1970). Methods have been described for measurement of (1) the photoreduction of ferredoxin and photoproduction

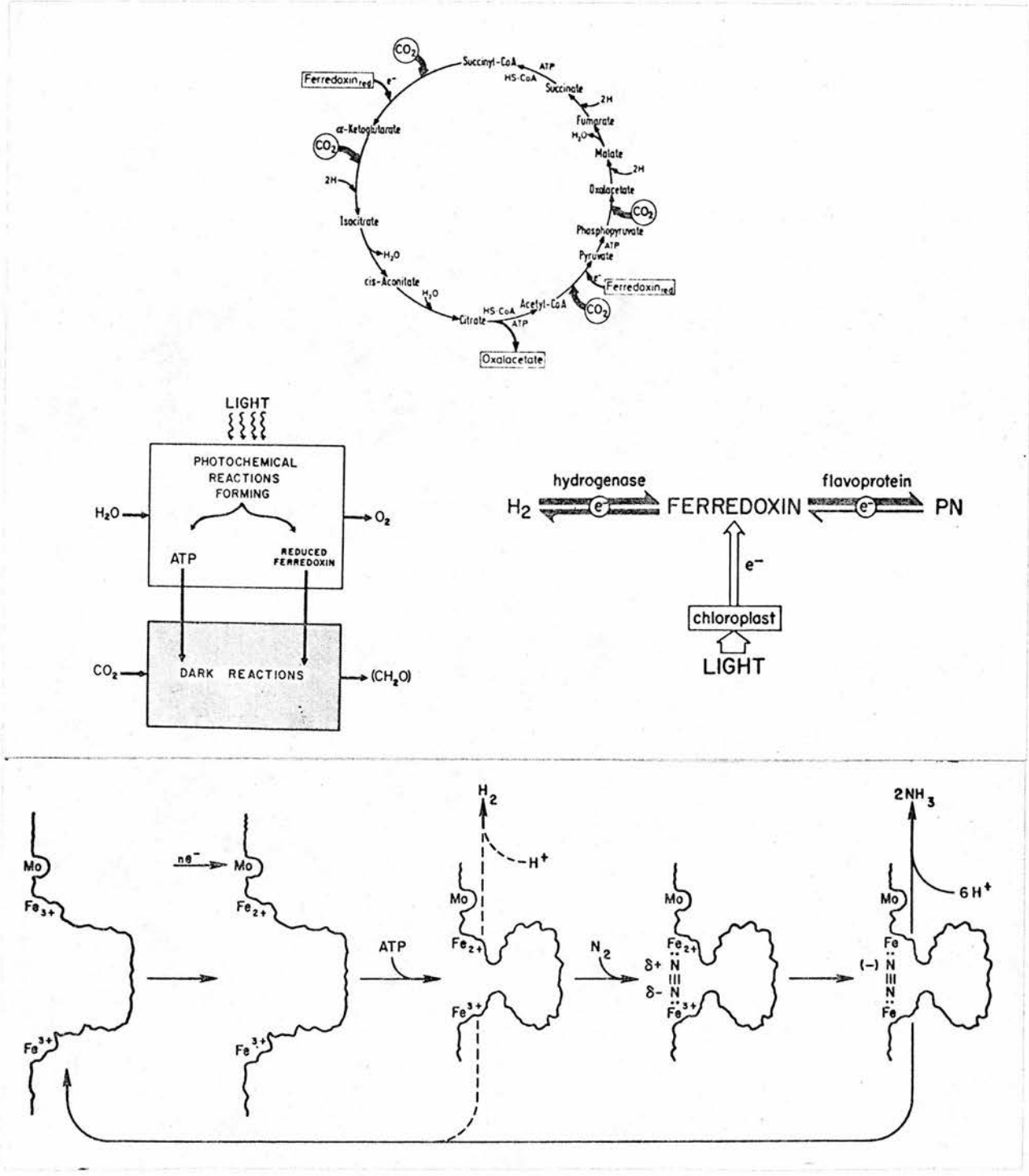


Fig. 8 Iron-sulphur protein-dependent biological systems

Above - The reductive carboxylic acid cycle in photosynthetic bacteria (from Arnon, 1969)

Centre left - Participation by ferredoxin in photosynthesis by chloroplasts (from Arnon, 1965b)

Centre right - Role of ferredoxin in the reduction of pyridine nucleotides (PN) in chloroplasts (from Arnon, 1965b)

Below - Hypothesis for the action of nitrogenase (from Bulen et al., 1965)

of oxygen (Arnon, Tsujimoto and McSwain, 1964), (2) the photoreduction of NAD and NADP (Bachofen and Arnon, 1966; Buchanan and Evans, 1969), and (3) photosynthetic phosporylation (del Campo, Ramirez and Arnon, 1968).

Ferredoxins also function in the conversion of pyruvate to acetyl phosphate (Akagi, 1965) for which assay conditions are described by Lovenberg, Buchanan and Rabinowitz (1963). Pyruvate and other keto acids are produced in bacteria by ferredoxin-dependent reactions, e.g. Propionyl-CoA + CO_2 + ferredoxin$_{red}$ $\longrightarrow$ α-ketobutyrate + ferredoxin$_{ox}$ + CoA (Buchanan, 1969).

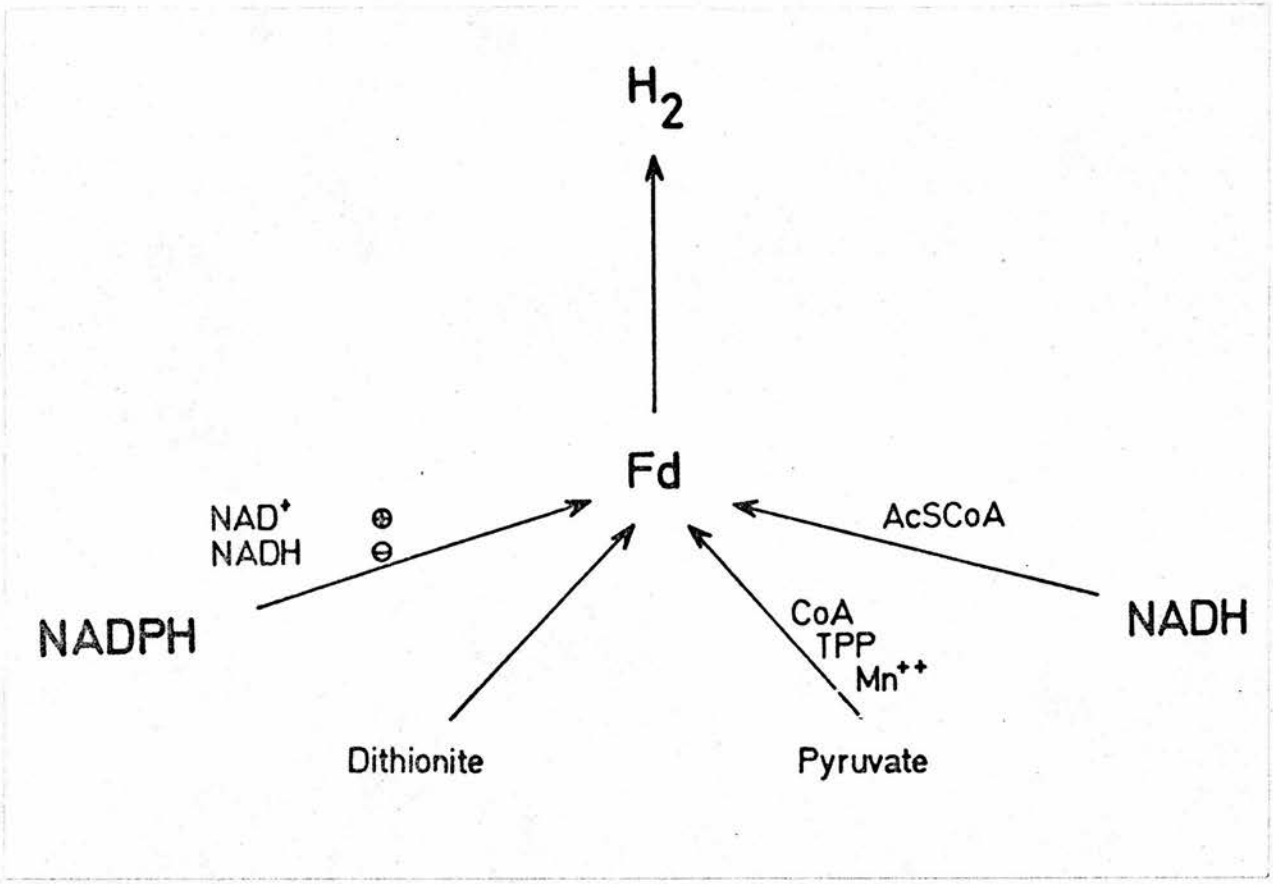
Ferredoxin "types" now recognised are (1) plant type (algae and higher plants)(Arnon, 1965a), (2) Clostridial or non-photosynthetic anaerobic bacterial type, e.g. Cl. pasteurianum, (3) green photosynthetic bacterial type, e.g. Chlorobium thiosulphatophilum (Gest, San Pietro and Vernon, 1963; Vernon, 1964), and (4) photosynthetic anaerobic bacterial type, e.g. Chromatium (Sasaki and Matsubara, 1967). The Chlorobium ferredoxin is chemically intermediate between Clostridium and Chromatium ferredoxins. However, despite the chemical dissimilarities these proteins are often functionally interchangeable e.g. Chlorobium ferredoxin can be photochemically reduced with isolated spinach chloroplasts (Buchanan, Matsubara and Evans, 1969).

Iron-sulphur proteins are also known to participate in nitrogen reduction and fixation (see San Pietro, 1965). The enzyme complex nitrogenase has been prepared from Azotobacter vinelandii (Bulen and LeComte, 1966), Cl. pasteurianum (Mortenson, Morris and Jeng, 1967), Klebsiella pneumoniae (Detroy et al., 1968), Bacillus polymyxa (Detroy et al., 1968) and Soya bean nodules (Klucas, Koch and Evans, 1968). It has at least five components (Taylor, 1969) of which one protein

contains Mo, Fe and labile sulphide and at least one other contains Fe and labile sulphide (Burns, Holsten and Hardy, 1970; DerVartanian, Shethna and Beinert, 1969). The nitrogenase-activity has been assayed by incubations using $^{15}N_2$ (Taylor, 1969), measuring reduction of acetylene to ethylene (Moustafa and Mortenson, 1968) and measuring the ATP-dependent evolution of hydrogen in the presence of sodium dithionite (Burns and Bulen, 1965). The enzyme (molybdoferredoxin) containing molybdenum, iron and labile sulphide has been obtained homogeneous and crystallised and has 2 Mo:34-38 Fe:26-28 labile sulphide atoms per molecular weight of 270,000 (Burns, Holsten and Hardy, 1970). The other protein which contains iron and labile sulphur but no molybdenum is called azoferredoxin and is as necessary for acetylene reduction as for nitrogen fixation (Moustafa and Mortenson, 1968).

The part ferredoxin plays in acetylene reduction (Kelly, 1969; Koch et al, 1970) and methane production (Wolfe, Wolin and Wolin, 1963) has been assessed and assays described. Iron-sulphur proteins are also concerned in the metabolism of hydrogen (see Fig. 9). Hydrogenase is also an iron-sulphur protein (Nakos and Mortenson, 1971a) and ferredoxin itself plays several roles in the metabolism of hydrogen in the organism. Valentine and Wolfe (1963) demonstrated an involvement of ferredoxin in the evolution of hydrogen from pyruvate, α-ketoglutarate, hypoxanthine and dithionite, while other aspects of ferredoxin-requirement for hydrogen metabolism have been described (Buchanan and Bachofen, 1968; Mortenson, 1964a, 1966). The reduction of ferredoxin by NADH, NADPH and pyruvate in Cl. kluyveri is required (1) for production of molecular hydrogen which in turn is involved in the production of ATP ($2H_2$/ATP) and (2) for the acetyl-CoA-dependent carbon dioxide fixation described earlier.

Fig. 9 Participation of iron-sulphur proteins in bacterial hydrogen metabolism



A. Hydrogen production and the ferredoxin of Cl. kluyveri (from Thauer et al., 1969)

1) H_2 ⟶ hydrogenase ⟶ ferredoxin ⟶ azoferredoxin and molybdoferredoxin (nitrogenase) ⟶ nitrogen fixation

2) dithionite ⟶ ferredoxin ⟶ hydrogenase ⟶ H_2

3) ? ⟶ rubredoxin ⟶ ?

B. Iron-sulphur proteins and hydrogen metabolism in Cl. pasteurianum (see Nakos and Mortenson, 1971a)

In this organism transhydrogenation reactions by ferredoxin reductase may be regulated by ferredoxin (Thauer et al., 1971). Ferredoxin-dependent reduction of NADP by hydrogen has been described (Valentine et al., 1963; Weaver, Tinker and Valentine, 1965). Sobel and Lovenberg (1966) found a systematic variation of the redox potential of ferredoxin with pH and suggested that this may provide a means of maintaining homeostasis with respect to H^+ ion which would be another important function of ferredoxins; but Tagawa and Arnon (1968) do not however agree with this hypothesis.

Other biological reductions in which ferredoxins play a part are those of hydroxylamine (Hewitt and Betts, 1963), nitrite (Losada et al., 1965; Huzisige and Satoh, 1961; Huzisige et al., 1963), xanthine (Valentine and Wolfe, 1963), uric acid (Valentine, Jackson and Wolfe, 1962) and sulphite (Akagi, 1965; LeGall and Dragoni, 1966). An effect of ferredoxin on initiation of sulphite oxidation has also been reported recently (Nakamura, 1970). The photochemical reduction of methaemoglobin is a characteristic of ferredoxin (Davenport, 1965).

Another haemprotein used as a substrate in the enzymatic assay of iron-sulphur proteins is cytochrome c (Omura et al., 1966; Peterson and Coon, 1968). Although it has not been suggested that the above reaction is of physiological importance, iron-sulphur proteins are thought to be concerned in transfer of electrons to haemproteins (notably cytochrome P450) for hydroxylation reactions. Hydroxylations in which independent iron-sulphur proteins are thought to play a role in electron-transfer include the ω-hydroxylation of hydrocarbons and fatty acids and fatty acid desaturation (Kusunose et al., 1968; Nagai and Bloch, 1966; Peterson and Coon, 1968), camphor hydroxylation (Cushman, Tsai and Gunsalus, 1967; Tsibris, Namtvedt and Gunsalus, 1968) and steroid hormone hydroxylations (Kimura and Suzuki, 1965;

Nakamura, Otsuka and Tamaoki, 1966; Omura et al, 1966). Huang and Kimura (1970) have in fact demonstrated that chemically reduced adrenodoxin supports steroid hydroxylations by adrenocortical cytochrome P450 in the absence of NADPH and adrenodoxin reductase. It is however generally accepted that a protein containing non-haem iron and labile sulphur does not participate in the hepatic microsomal hydroxylations of steroids and other substrates and indeed such proteins are probably absent in the liver (e.g. see Kimura and Ohno, 1968). A similar situation is believed to occur in adrenocortical microsomal hydroxylations (e.g. Masters et al, 1971; Sweat et al, 1969). Adrenodoxin-like proteins are thought however to participate in steroidogenic hydroxylations by the mitochondria of ovaries (Sulimovici and Boyd, 1968b, 1969), placenta (Mason and Boyd, 1971) and testes (Kimura and Ohno, 1968). Kimura and Ohno (1968) also confirmed that the testis and ovary ISP could substitute for adrenodoxin in a DOC 11β-hydroxylating activity assay while corresponding preparations from liver, and Euglena and spinach ferredoxins, Pseudomonas rubredoxin and Pseudomonas putidaredoxin were inactive. Omura et al.(1967) also noted that adrenodoxin and spinach ferredoxin are not functionally interchangeable. Kimura (1968) states that adrenodoxin-like proteins are not found in brain, heart, liver, kidney and pancreas and notes the point that these proteins appear to be confined to glands concerned in the biosynthesis of steroid hormones. This raised the question of the possibility of a functional significance in this distribution (see Table I).

Purification of iron-sulphur proteins and their stability: A variety of methods have been described for the purification of iron-sulphur proteins. In most cases the steps of purification involve ammonium sulphate fractionation, ion-exchange column chromatography, gel-filtration, preparative electrophoresis, fractionation using

organic solvents etc. However in view of the variety of tissues used as starting materials and the variety of functions which these enzymes undertake (thereby resulting in diverse conditions to which individual proteins are found to be labile) no common scheme of purification of "ferredoxins" has been evolved.

A first consideration in deciding on the method of purification to be employed is whether the iron-sulphur protein is the only enzyme to be recovered from the tissue or whether another enzyme such as its reductase (usually a flavoprotein) and(or) an associated haemprotein (such as cytochrome P450) is to be conserved as well. For chemical studies where the iron-sulphur protein alone would suffice the rest of the enzyme preparation can be discarded at each step of purification. This approach considerably reduces the work entailed at each stage and the time expended to reach a required degree of purity in addition to sometimes increasing the yield of enzyme both by permitting a larger portion of each protein fraction to be taken as well as hastening the processes in which the enzyme is less stable. However in view of the fact that in most cases a multicomponent enzyme system is concerned, often other enzymes are required to be isolated both for the assay of the ISP and for reconstitution and study of the biological activity. Specific examples are the methods for preparation of spinach ferredoxin (San Pietro, 1963; Tagawa and Arnon, 1962) compared with the combined preparation of spinach ferredoxin and ferredoxin-NADP reductase as developed by Borchert and Wessels (1970), and again the isolation of adrenodoxin alone (Kimura, 1968) and adrenodoxin and its associated flavoprotein and haemprotein (Omura et al, 1967).

Some features are comparable in the preparation of iron-sulphur proteins. Many of them have a considerable number of acidic amino

acids in the molecule (see Table III) resulting in a low isoelectric point and a tight binding to DEAE-type anion exchangers. This characteristic is of considerable help in desorbing more weakly bound impurities from anion exchangers before iron-sulphur proteins and Kimura (1968) has employed this property to adsorb adrenodoxin onto an anion exchanger from a relatively crude enzyme preparation.

Due to the lability of iron-sulphur proteins to acid conditions care has to be taken in preparation to see that fractionating reagents (e.g. ammonium sulphate) are adequately buffered. Phosphate buffer however is reported to "bleach" the protein preparation by leaching out iron from bacterial ferredoxins (Mortenson, 1963) and from adrenodoxin (Omura et al., 1967). Cold is reported to inactivate azoferredoxin (Moustafa and Mortenson, 1968) and freezing to harm adrenodoxin (Omura et al., 1965a). In this connection, heating ferredoxins obtained from the thermophilic bacteria, Cl. tartarivorum and Cl. thermosaccharolyticum, to 70°C is far less harmful than the effect on ferredoxins from mesophilic clostridia, and storing freeze-dried thermophile ferredoxins at -20°C is less harmful than storing them in solution at 4°C or -20°C. The observations on the comparative heat stabilities of these ferredoxins are attributed to different environments around the iron and labile sulphide (Devanathan et al., 1969). Taylor (1969) has described the different heat-stable and heat-labile fractions of a clostridial nitrogen-fixing extract. Mitsui (1970) reports that the enzymatic activity of crystalline Euglena and Chlamydomonas ferredoxins remained intact after one year in ammonium sulphate at pH 8.0 at 4°C and was still present after 3 years.

A final commonly observed factor which affects the stability of iron-sulphur proteins is dissolved oxygen. Dissolved oxygen is known

not only to reoxidise a reduced chromophore (Kimura and Suzuki, 1967; Omura et al, 1967) but to denature several iron-sulphur proteins as well. Thus hydrogenase is readily inactivated by oxygen or air but may be stored for months under hydrogen at 0-5°C (Nakos and Mortenson, 1971a) and azoferredoxin from Cl. pasteurianum may also be prepared and stored under hydrogen although it is extremely oxygen-sensitive (Moustafa and Mortenson, 1969). Several described methods for the preparation and storage of iron-sulphur proteins suggest working solutions made anaerobic with hydrogen, nitrogen or argon: two examples are the method for purification of molybdoferredoxin described by Burns, Holsten and Hardy (1970) and of adrenodoxin by Orme-Johnson and Beinert (1969c).

Representative methods of preparing various types of iron-sulphur proteins are described in the preceding references while the preparation of clostridial ferredoxin has been described by Buchanan, Lovenberg and Rabinowitz (1963) and Lovenberg and Sobel (1965) and rubredoxin by Lovenberg and Williams (1969). An isolation procedure of ferredoxin from a green alga, Scenedesmus, is described by Sugeno and Matsubara (1968), the leaves of a tree, Leucena glauca, by Benson and Yasunobu (1969a) and cotton seedling cotyledons by Newman, Ihle and Dure (1969). The preparation of testis iron-sulphur protein was described by Kimura and Ohno (1968) and additional details on the behaviour of adrenodoxin during purification are found in Suzuki and Kimura (1965).

Comparison of light extinction at characteristic wavelengths as a criterion of purity: Ascertaining the state of purity of an electron-transporting non-haem iron protein, particularly in the course of purification when a relatively rapid and economical method of assessment is desirable, presents certain problems. The rubredoxins in

general possess no known enzymatic function which can be assayed while the ferredoxins need usually to be coupled with at least a flavoprotein and often a haemprotein, cytochrome P450 or cytochrome c, in order to obtain a value for its electron-transfer / activity. As will be described later in the present work, the conditions of the assay media used (in particular NADH or NADPH concentration, ionic strength and protein concentration) markedly influence the rates of electron-transfer observed in addition to the more usual parameters like the temperature of the reaction. Furthermore when dealing with a multi-component system (e.g. estimating the flavoprotein-adrenodoxin complex of mitochondrial cytochrome P450 reductase by measuring the rate of cytochrome c reduction) doubling the input of a two-component reductase increases the velocity of the reaction by the square (Omura et al, 1966). It has also been observed that addition of a large excess of iron-sulphur protein in the presence of fixed amounts of the other components can lead to a decrease of enzymic reaction rate by inhibiting electron-flow (this work; Nelson and Neumann, 1968). These factors will lead to expenditure of time in adjusting incubation conditions (e.g. by dialysis at each stage of purification) and excessive consumption of purified enzyme to arrive at an activity value which may not be meaningful.

A common practice adopted to follow the course of purification of an iron-sulphur protein is therefore to measure its optical extinction at a given characteristic wavelength in the visible range and its "protein extinction" at a stated wavelength in the ultraviolet and calculate the ratio of the first to the second. This value is quite unsuitable as an absolute guide to purity and cannot replace established criteria of electrophoresis, gel-filtration, ion-exchange chromatography, amino acid analysis etc. in this respect.

However once the preparation reaches a stage (which may be judged by spectrophotometric scan of the optical spectrum) where only the desired protein contributes to the extinction at the chosen wavelength in the visible spectrum, the concept of ascertaining the ratio of extinction at two characteristic wavelengths is a useful, rapid and economical technique of following the course of purification of iron-sulphur proteins. In the case of some iron-sulphur proteins such as adrenodoxin (Fig. 11) the method is particularly useful as the molar extinction at 280 nm is unusually low due to a very low content of aromatic amino acids (Kimura et al, 1969).

Values of this type of ratio obtained for some purified iron-sulphur proteins are as follows: Cl. pasteurianum ferredoxin - $E^{390nm}:E^{280nm} = 0.83$, Cl. acidi-urici ferredoxin - $E^{390nm}:E^{280nm} = 0.789$ (Hong and Rabinowitz, 1970a); spinach and alfalfa ferredoxin - $E^{422nm}:E^{277nm} = 0.49$ (Borchert and Wessels, 1970; Keresztes-Nagy and Margoliash, 1966); M. aerogenes rubredoxin - $E^{280nm}:E^{490nm} = 2.5$ (Bachmeyer et al, 1968a); putidaredoxin - $E^{415nm}:E^{277nm} = 0.32$ (Cushman, Tsai and Gunsalus, 1967); adrenodoxin - $E^{414nm}:E^{280nm} = 0.76$ (Kimura and Suzuki, 1967). It should be noted that these values could be affected by the presence of other proteins having an extinction at either the optical or ultraviolet wavelengths chosen or by loss of the labile iron-sulphur chromophore of the non-haem iron protein since the apoprotein has a much lower extinction in the visible region of the spectrum (Hong and Rabinowitz, 1970a). In a later section will be discussed reconstituted (Hong and Rabinowitz, 1970b; Kimura, 1968) and "artificial" ISP^s which, while possessing "typical" optical extinction spectra and containing iron and labile sulphur, often have lessened or no biological activity.

Properties of iron-sulphur proteins

Optical and ultraviolet extinction spectra: Fig. 10 gives the extinction characteristics of some representative members of this class of proteins. Typically there is a single or double peak of light extinction at about 400-500 nm and at about 370-390 nm and ultraviolet extinction as in many other proteins. Reduction of ISP^s either physiologically or chemically as with dithionite usually results in the bleaching of the chromophore to various extents. Fig. 10 shows the effect of reducing Chromatium ferredoxin with hydrogen, sodium dithionite and light respectively (R. Bachofen and D. I. Arnon in Arnon, 1965b). Preparation of apoproteins by chemical methods results in bleaching but the extinction spectra can be reconstituted (Hong and Rabinowitz, 1967; Kimura, 1968; Malkin and Rabinowitz, 1966a). The visible extinction appears to be directly linked to the iron-sulphur chromophore. Fig. 11 gives the visible and ultraviolet spectral characteristics of adrenodoxin (Kimura and Huang, 1970).

Extinction coefficients have been determined for several iron-sulphur proteins; Clostridial ferredoxin (Hong and Rabinowitz, 1970a; Palmer, Sands and Mortenson, 1966), parsley ferredoxin (Bendall, Gregory and Hill, 1963), Scenedesmus ferredoxin (Matsubara, 1968), Pseudomonas rubredoxin (Peterson and Coon, 1968). Of the several others reported reference may be obtained from San Pietro (1965).

Extinction coefficients at several wavelengths have been reported for native adrenodoxin and apoadrenodoxin (Kimura, 1968). The enzymatic reduction of the adrenodoxin chromophore and the slow reoxidations of the chromophore on reintroduction of air have been described by Estabrook (1966), Kimura and Suzuki (1965, 1967) and Omura et al. (1967).

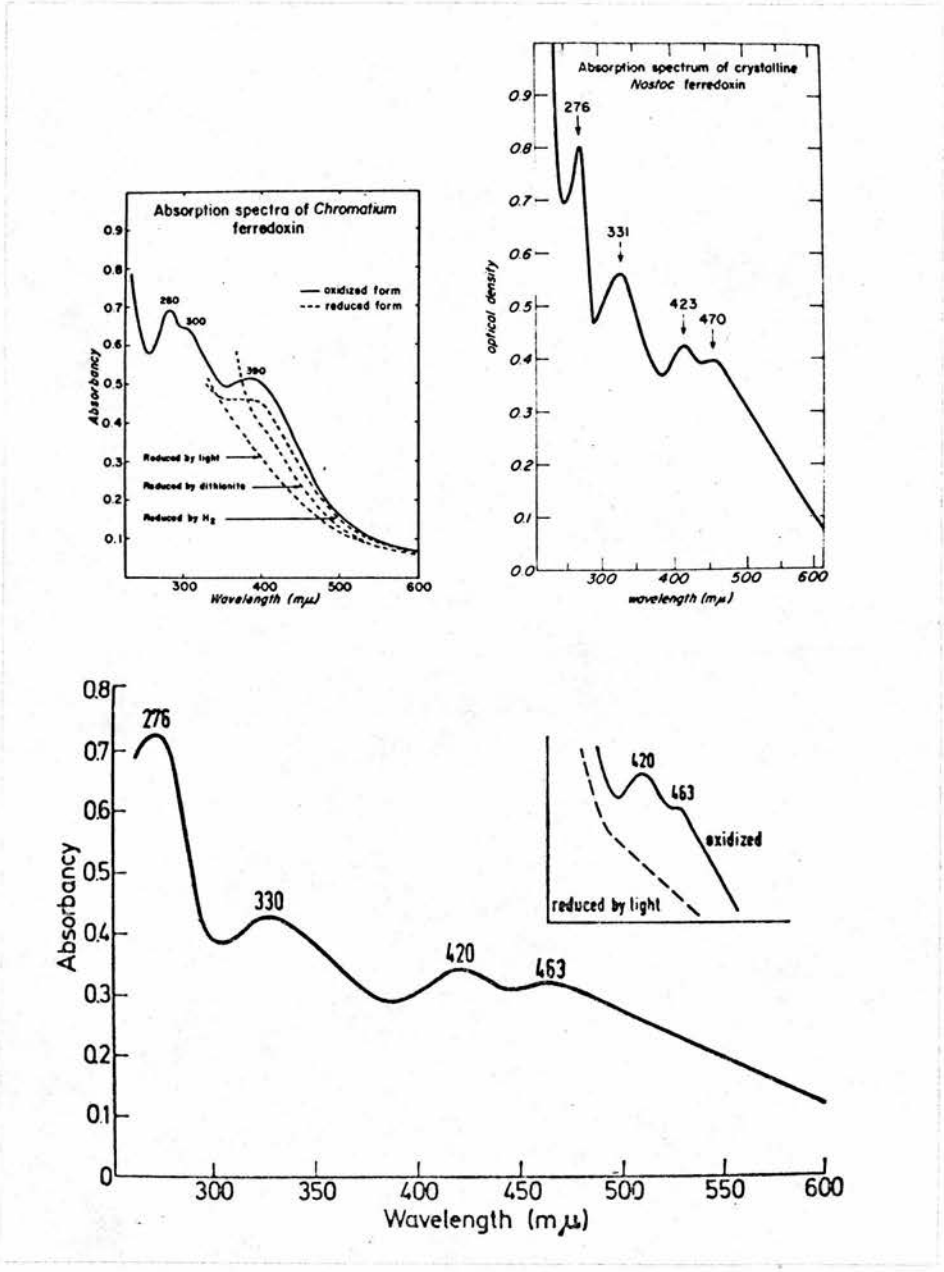


Fig. 10 Spectral properties of some ferredoxins

Above left - Chromatium ferredoxin (Bachofen and Arnon in Arnon, 1965b)

Above right - Nostoc ferredoxin (Mitsui and Arnon in Arnon, 1965b)

Below - spinach ferredoxin (from Arnon, 1969)

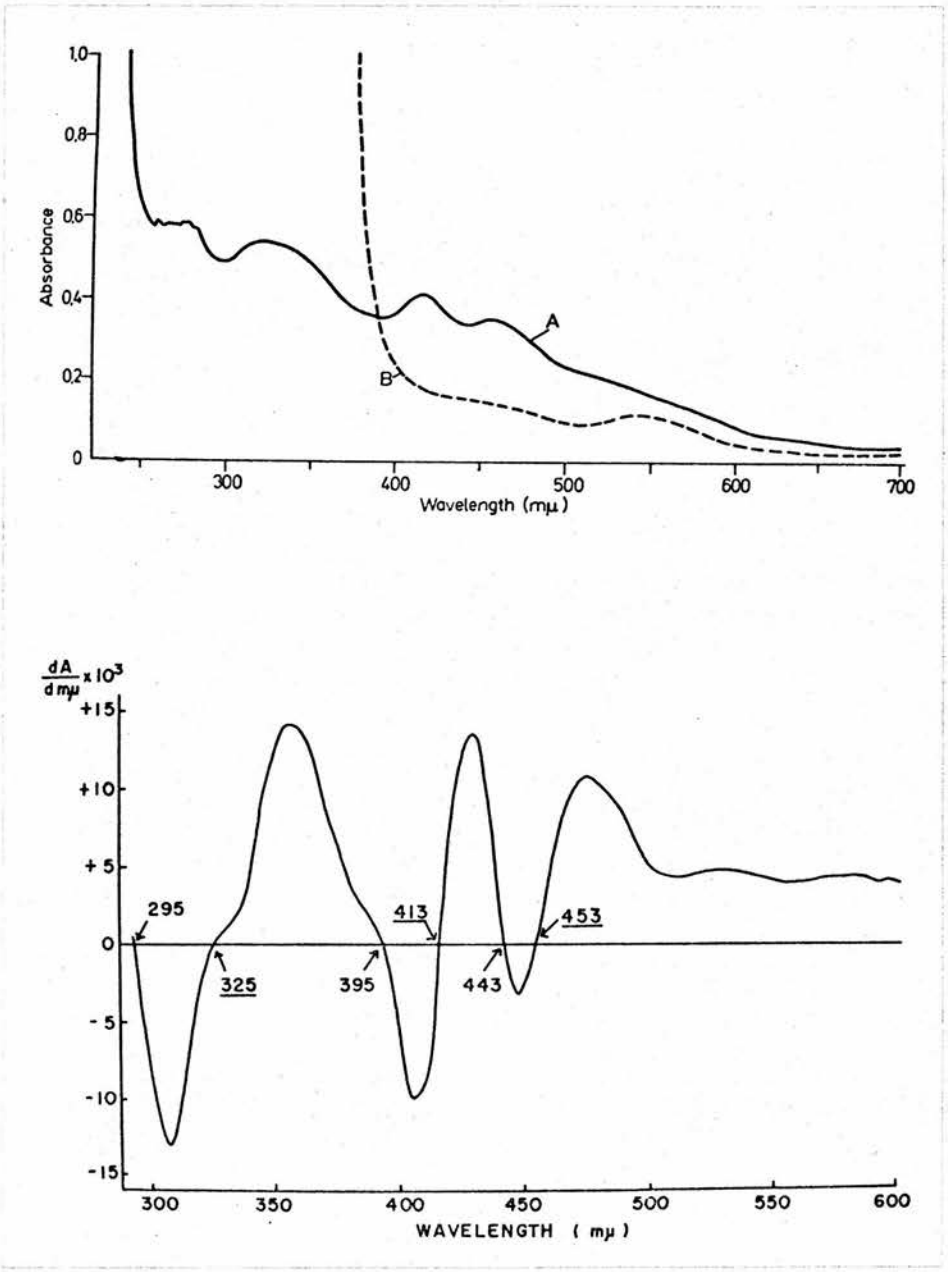


Fig. 11 Spectral characteristics of adrenodoxin

Above - Absolute spectra (curve A - oxidised form; curve B - after reduction with dithionite) (from Kimura, 1968)

Below - Differential spectrum of the oxidised form (the difference of wavelength was 1.0 nm) (from Kimura and Huang, 1970)

Infrared extinction spectra: Less work has been done on this portion of the spectrum of ISP^s than on the rest. Lovenberg, Buchanan and Rabinowitz (1963) and Buchanan, Lovenberg and Rabinowitz (1963) have described this for clostridial ferredoxin and McCarthy and Lovenberg (1968) have published the infrared extinction characteristics of the three artificial iron-sulphur proteins derived from bovine serum albumin.

The infrared spectral characteristics of adrenodoxin have however been well defined by Kimura et al.(1969) and Kimura and Huang (1970).

The use of "physical" techniques to examine biological material has made rapid advances in recent years and a certain amount of work has been done on ISP^s as well.

Electron paramagnetic resonance (EPR) spectroscopy: Beinert (1965) describes the initial stages in the use of EPR to examine ISP^s "of the mitochondrial type" (such as NADH dehydrogenase, succinic dehydrogenase). This work demonstrated that the large amounts of iron chemically demonstrated earlier in submitochondrial fractions but not forming part of the haem content were not due to preparative contamination but were probably associated with the proteins which at low temperature and in reducing conditions gave rise to a strong characteristic EPR signal at $g = 1.94$. Although the $g = 1.94$ (approx.) signal was at first considered not to be a characteristic of ferredoxins (as opposed to other iron-sulphur proteins) (Beinert, 1965) later work (Hall, Gibson and Whatley, 1966; Palmer and Sands, 1966; Palmer, Sands and Mortenson, 1966) demonstrated convincingly that reduced spinach and clostridial ferredoxins at liquid helium temperatures gave EPR signals at $g = 1.89$, 1.96 and 2.04 and $g = 1.89$, 1.96 and 2.00 respectively. Of the considerable use of EPR made to study iron-sulphur proteins recently may be mentioned results of Orme-

Johnson and Beinert (1969a) showing that during anaerobic reductive titration of clostridial ferredoxin and milk xanthine oxidase two different EPR signals arise in succession. The authors suggest that this indicates that more than one species of iron is involved in electron transfer in these proteins. EPR spectra of native (Fe^{56}) and Fe^{57}-substituted enzymes have revealed hyperfine splitting which yields evidence on interaction between individual iron atoms and between iron and sulphur atoms in a single protein molecule. Such evidence has been obtained for putidaredoxin (Cushman, Tsai and Gunsalus, 1967; Tsibris et al, 1968) and adrenodoxin (Beinert and Orme-Johnson, 1969; Orme-Johnson, Hansen and Beinert, 1968). EPR studies on certain ISP^s have demonstrated that those proteins (viz. an iron-sulphur protein from Cl. pasteurianum, putidaredoxin, an A. vinelandii protein and bovine and porcine adrenodoxins) contain two or more Fe atoms per protein molecule. When the Fe^{57}-EPR spectra were sufficiently resolved, these studies also showed that the signal at $g = 1.94$ involves a binuclear Fe complex accepting one electron (Orme-Johnson, Hansen and Beinert, 1968). Similar studies indicate that the relationship of iron atoms to each other may be different in spinach (Palmer, 1967) and clostridial (Orme-Johnson and Beinert, 1969a) ferredoxins. Tsibris and Woody (1970) and Kimura (1968) describe various attempts using EPR to probe the structure of iron-sulphur proteins.

Omura et al.(1965b) described the EPR signal ($g = 1.94$) of adrenodoxin and a detailed EPR study of the protein was published subsequently (Watari and Kimura, 1966). The signal can be obtained by reduction with dithionite (Omura et al, 1965b) or with NADPH and adrenodoxin reductase and is lost on reoxidation by aeration (Watari and Kimura, 1966). The g values for adrenodoxin are 1.94 and 2.01 and

the signal intensity is markedly dependent on temperature (Watari and Kimura, 1966). Cammack, Rao and Hall (1971) showed that low concentrations of certain solubilising or "chaotropic" agents made the EPR spectrum of spinach ferredoxin approach that of adrenodoxin. Studies on the (reduced) related testis protein, testodoxin, showed a temperature-sensitive EPR signal at g = 1.92 and 2.02 (Kimura and Ohno, 1968).

In conclusion the EPR studies help to confirm chemical determination of iron valency in ISP^S (Shethna et al., 1964). Bacterial rubredoxins (Lovenberg, 1966) are thought to have iron in the ferric state and give an EPR signal at g = 4.30 (at the temperature of liquid nitrogen) which is lost on reduction with dithionite. EPR and chemical studies have also led to the suggestion that one of the two atoms of iron in adrenodoxin exists in a more easily reducible state than the other (Kimura and Suzuki, 1967; Watari and Kimura, 1966).

Nuclear magnetic resonance (NMR), Mossbauer spectroscopy and magnetic susceptibility measurements: Early studies of NMR and Mossbauer spectroscopy of iron-sulphur proteins have been described by Phillips, Knight and Blomstrom (1965) and Bearden et al.(1965). Further Mossbauer studies on spinach ferredoxin, Chromatium ferredoxin and Chromatium HiPIP were published by Bearden and Moss (1966). Detailed Mossbauer studies on Cl. pasteurianum ferredoxin (Blomstrom et al., 1964), spinach ferredoxin (Johnson et al., 1969; Moss et al., 1968), Chromatium ferredoxin (Moss et al., 1968), Euglena ferredoxin (Johnson et al., 1968), Scenedesmus ferredoxin (Rao et al., 1971) and putidaredoxin (Cooke et al., 1968) have been made. The data obtained give valuable information for the formulation of theories regarding the iron atoms and the active-centre which will be discussed later.

Proton magnetic resonance and magnetic susceptibility comparison of clostridial ferredoxins show striking similarity in the magnetic, electronic and geometrical characters of the iron-sulphur centres of these proteins (Poe et al., 1970, 1971a). These properties have been recently compared in Cl. pasteurianum ferredoxin and rubredoxin (Phillips et al., 1970).

Detailed studies on the magnetic susceptibility of bovine adrenodoxin have been published (Kimura, Tasaki and Watari, 1970). These studies (over the temperature range 4^{o}-260^{o}K) showed that oxidised adrenodoxin is diamagnetic and the reduced form is paramagnetic as are spinach and parsley ferredoxins (Moss, Petering and Palmer, 1969). The diamagnetism of oxidised adrenodoxin ~~is in~~ accounts for the loss of EPR signal.

Circular Dichroism (CD): The CD characteristics have been described for iron-sulphur proteins (Coffman and Stavens, 1970; Eaton and Lovenberg, 1970; Newman and Postgate, 1968; Palmer et al., 1966; Peterson and Coon, 1968) and CD studies of the complex between ferredoxin and ferredoxin reductase have been made (Cammack et al., 1971).

The circular dichroism of adrenodoxin and related mammalian proteins has been thoroughly investigated by Kimura's group (Kimura, 1968; Kimura et al., 1969; Kimura and Ohno, 1968; Padmanabhan and Kimura, 1970). The spectrum (Kimura et al., 1969) shows a minimum at 228 nm compared to 219 nm for spinach ferredoxin and the cross-over points are at different wavelengths as well. This may be due to differences in structure between the two proteins. The changes in circular dichroism during controlled denaturation of the chromophore of adrenodoxin have been studied (Padmanabhan and Kimura, 1970).

Optical Rotatory Dispersion (ORD) and Cotton effects: The use of ORD to examine the structure of some iron-sulphur proteins has been described by Ulmer and Vallee (1963) and Vallee and Ulmer (1965) and extended for several other members of the same class (DerVartanian, Shethna and Beinert, 1969; Garbett et al., 1967; Gillard et al., 1965, 1966; Lovenberg and Williams, 1969; Peterson and Coon, 1968). That the effects of "chaotropic" agents on the ORD spectrum of spinach ferredoxin do not correlate directly with the effects on the EPR spectrum has been reported recently by Cammack, Rao and Hall (1971).

Kimura and coworkers have studied the ORD spectra and Cotton effects of adrenodoxin and related proteins (Kimura and Ohno, 1968; Kimura and Suzuki, 1967; Kimura et al., 1969). The optical rotatory dispersion of adrenodoxin and spinach ferredoxin show similar changes on reduction so that it was suggested that the protein ligands in iron binding and steric organisation of the two proteins were similar (Kimura and Suzuki, 1967; Kimura, 1968). However from the difference in ultraviolet ORD and other measurements it is also possible that whereas spinach ferredoxin has some β-helix, adrenodoxin may have some α-helix (Kimura et al., 1969). This may be due to the difference in prolyl residues between these proteins and may account for the inability, notwithstanding other similarities, of several other ISP^s to substitute for the biological activity of adrenodoxin (Kimura and Ohno, 1968).

Magneto-Optical Rotatory Dispersion (MORD): Marlborough, Hall and Cammack (1969) have studied the ORD of spinach ferredoxin in the presence and absence of a 3 kilogauss magnetic field. This has given further information on the differences between the oxidised and reduced forms and on phenomena related to the decomposition of the protein.

Laser-Raman spectroscopy: Studies on the iron coordination of Cl. pasteurianum rubredoxin have been made recently using this technique on solid amorphous non-crystalline Cl. pasteurianum rubredoxin and crystallised protein (Long and Loehr, 1970) and the same material in aqueous solution (Long et al., 1971).

Other related studies on adrenodoxin and other iron-sulphur proteins: Mildvan, Estabrook and Palmer (1966) have studied the effect of spinach ferredoxin and adrenoxin on the relaxation rates of the protons of water. Their results indicated that the paramagnetic centres in ferredoxin and adrenodoxin are rather inaccessible to protons and accessibility is slightly increased upon reduction.

Shape of ISP^S - frictional ratio and axial ratio: Appella and San Pietro (1962) used data from viscosity, sedimentation and diffusion determinations to estimate that the axial ratio of plant chloroplast PPNR (ferredoxin) was between 14 and 18. They noted that the value was high for such a low molecular weight protein.

Kimura et al.(1969) have defined these values for bovine adrenodoxin. They found the frictional ratio (f/fo) was about 1.29 and the axial ratio (a/b) about 5, which suggested that the protein was a globular molecule. This was in accordance with values obtained for the intrinsic viscosity.

Intrinsic viscosity: Viscosity determinations on chloroplast PPNR (Appella and San Pietro, 1962) gave a reduced viscosity value of 0.150 dl/g at zero concentration.

The intrinsic viscosity of bovine adrenodoxin computed by Kimura et al (1969) from experimental data was 3.0 ml/g.

Diffusion Coefficient: This characteristic has been determined for a number of iron-sulphur proteins. Some published values are: chloroplast PPNR - $6.6 \times 10^{-7} cm^2 sec^{-1}$ (Appella and San Pietro, 1962);

Pseudomonas oleovorans rubredoxin - 11.7 x $10^{-7}cm^2sec^{-1}$ (Peterson and Coon, 1968); alfalfa ferredoxin - 10.39 x $10^{-7}cm^2sec^{-1}$ (Keresztes-Nagy and Margoliash, 1966) and clostridial paramagnetic protein - 10.2 x $10^{-7}cm^2sec^{-1}$ (Hardy et al., 1965).

The measurement for bovine adrenodoxin has been described (Kimura et al., 1969) to be 11.2 x $10^{-7}cm^2sec^{-1}$, which is higher than an earlier assumption for porcine adrenodoxin (Suzuki and Kimura, 1965).

Sedimentation coefficient: This parameter has been defined for a considerable number of iron-sulphur proteins in view of its value in determining molecular weights. Values for some members of the class are: chloroplast PPNR - 1.36 S (Appella and San Pietro, 1962); P. oleovorans rubredoxin - 1.66 S (Peterson and Coon, 1968); alfalfa ferredoxin 1.58 S (Keresztes-Nagy and Margoliash, 1966); bacteroid ISP - 1.3 S (Koch et al., 1970) and clostridial paramagnetic protein - 2.53 S (Hardy et al., 1965). The sedimentation coefficient of chloroplast PPNR appeared to vary with protein concentration and may have indicated some association (Appella and San Pietro, 1962) while plots of concentration of the Azotobacter iron-sulphur proteins during sedimentation equilibrium against a range of concentrations gave straight lines (DerVartanian, Shethna and Beinert, 1969).

Adrenodoxin is reported to have a sedimentation coefficient of 1.55 S (Kimura and Suzuki, 1967) and more detailed sedimentation equilibrium studies by three different methods showed that there was no tendency of variation of molecular weight with protein concentration of adrenodoxin (Kimura et al., 1969). Testis iron-sulphur protein has a sedimentation coefficient of 1.9 S (Kimura and Ohno, 1968).

Partial specific volume: This has been experimentally determined or calculated for several iron-sulphur proteins including alfalfa ferredoxin - 0.715 ml/g (Keresztes-Nagy and Margoliash, 1966);

parsley ferredoxin - 0.71 ml/g (Bendall, Gregory and Hill, 1963); Azotobacter iron-sulphur proteins - 0.74 ml/g (DerVartanian, Shethna and Beinert, 1969) and Cl. pasteurianum ferredoxin - 0.63 ml/g (Lovenberg, Buchanan and Rabinowitz, 1963). Devanathan et al.(1969) reported that in two other clostridial ferredoxins the partial specific volume of the native protein was 0.62 ml/g and the apoprotein 0.70 ml/g; the experimental values agreeing well with those calculated.

Kimura et al.(1969) find an experimental value for the partial specific volume of bovine adrenodoxin of 0.70 ml/g at 20°C which is close to the theoretical value of 0.72 ml/g calculated from the amino acid composition.

Electrophoretic mobility: Keresztes-Nagy and Margoliash (1966) attempt to define some of the characteristics of the electrophoresis of alfalfa ferredoxin. They describe it as having unusually high anionic mobility at pH 7.2 and find that the native protein moves faster than the colourless deteriorated material. Some velocities are stated. Different salt compositions of the medium although at the same ionic strength appear to affect the electrophoretic mobility of the ferredoxin.

Effect of temperature: Systematic examination of the effects of temperature change on ferredoxin-like proteins have not been commonly undertaken except in the course of measurement of EPR etc. As might have been predicted ferredoxins from thermophilic bacteria are much more stable to heat than those from mesophiles. Devanathan et al. (1969) discuss possible reasons for the higher temperature stability of these ferredoxins.

Kimura and Huang (1970) have also examined the optical spectrum down to 300 nm of oxidised bovine adrenodoxin in 50% glycerol solution at the temperature of liquid nitrogen. Their observations of a slight

shift in extinction wavelengths and line sharpening corroborate earlier reports (Palmer, Brintzinger and Estabrook, 1967).

Isoelectric points and pH optima: Table III includes figures for the sum of glutamic and aspartic residues per molecule for a number of ISP[s]. This indicates the high proportion of acidic residues present in these proteins, thus accounting for their tight binding to DEAE-cellulose as described earlier. The abundance of acidic residues leads to a low isoelectric point although very precise investigation of this characteristic is difficult to make experimentally since the iron-sulphur chromophore of the native protein is readily lost at acid pH/. Of published values located in the literature: bacteroid ISP gave a single band at pH 6.0 but the enzymatic activity was by then largely lost (Koch et al, 1970); an extrapolation of electrophoretic mobility at different pH values gave an isoelectric point of 3.7 for Cl. pasteurianum ferredoxin (Lovenberg, Buchanan and Rabinowitz, 1963) and 5.0 for clostridial hydrogenase (Nakos and Mortenson, 1971a).

Due to the lability of ferredoxin to acid pH and due to its association with another protein for its enzymatic activity not much is described as to pH optima for activity (Buchanan, Evans and Arnon; 1965; San Pietro and Lang, 1958).

Redox potentials: The redox potentials of the "typical" iron-sulphur proteins are of considerable interest in view of the fact that they are close to the potential of the hydrogen electrode ($\mathcal{E}^{o}$ = -0.42 V). Table III lists published redox potentials of several proteins. It is seen that whereas the two (Chromatium and Rhodopseudomonas) HiPIP[s] have potentials around +0.35 V, and rubredoxins have potentials around -50 mV the ferredoxins appear to have potentials closer to -0.4 V. The published values are those most accepted today but the

difficulties in obtaining an accurate figure are considerable.

Earlier estimates of a redox potential of +0.15 V for adrenodoxin (Kimura, 1968) were lowered to -0.164 V and now are accepted as being nearer -0.37 V. This will be discussed later in further detail.

Number of electrons transferred per molecule: This characteristic is related to that of the redox potential and has been also the subject of some contradictory findings. Table III gives the values commonly accepted today. This work has been facilitated by the development of the EPR technique as described by Beinert and Orme-Johnson (1969) and Orme-Johnson and Beinert (1969b). The major recent publications on this important aspect of electron transport in iron-sulphur proteins are by Evans et al.(1968), Mayhew et al.(1969) and Orme-Johnson and Beinert (1969c). Rao et al.(1971) have used Mossbauer spectroscopy to confirm anti-ferromagnetically coupled iron atoms and to investigate electron transfer in plant ferredoxins.

Despite the recent interest on the stoichiometry of electron transfer by ISP^S contradictory evidence was found in the literature regarding adrenodoxin. By different experimental methods it had been found that adrenodoxin appeared to transfer one reducing equivalent according to Orme-Johnson and Beinert (1969c), and two reducing equivalents according to Kimura and Suzuki (1967) and Kimura et al.(1967). In the present work this was examined further.

Induction and repression characteristics: Not very much is known about the induction and repression of ferredoxins. Mitsui (1970) reports that citrate added to the culture medium of Chlamydomonas markedly suppresses ferredoxin formation while this enzyme was also not obtained from dark cultures of Euglena. Azoferredoxin and molybdoferredoxin which are components of the nitrogen fixing systems of Cl. pasteurianum are only synthesised by the cells when the ammonium

ion content of the medium is limiting or low (Jeng, Devanathan and Mortenson, 1969). This may apply to the system in Azotobacter vinelandii as well (Bulen and LeComte, 1966).

Kowal (1970) found that ACTH induced an increase in the adrenodoxin content of adrenal cortex cells.

Cell-free synthesis: Nepokroeff and Aronson (1970) report the synthesis of ferredoxin in vitro using labelled amino acids and cell-free systems from Cl. pasteurianum. Bayer, Jung and Hagenmaier (1968) have described the solid-phase synthesis of the amino acid sequence of Cl. pasteurianum ferredoxin.

Immunological studies: Nitz et al.(1969) have described studies using rabbit antisera to native ferredoxin and derivatives. Hong and Rabinowitz (1970c) have also made studies in this field using clostridial ferredoxins and apoferredoxins in spite of earlier difficulties (Lovenberg, Buchanan and Rabinowitz, 1963) in obtaining antibodies to these low molecular weight proteins. Hiedemann-van Wyk and Kannangara (1971)have determined the localisation of spinach ferredoxin in the thylakoid membrane using immunological methods.

Masters et al.(1971) have used immunochemical techniques to prove the absence of adrenodoxin-like proteins in hepatic and adrenocortical microsomes.

Crystals and x-ray crystallography: The crystallisation of a variety of iron-sulphur proteins has been accomplished from the first few years of work on these enzymes (Buchanan and Rabinowitz, 1964; Lovenberg, 1966; Lovenberg, Buchanan and Rabinowitz, 1963 and Tagawa and Arnon, 1962). The published microphotographs show that the crystals obtained have a variety of forms. More recent crystallisations of ISP^S are described in Bachmeyer et al.(1968a); Burns, Holsten

and Hardy (1970); Hong and Rabinowitz (1970b) and Mitsui (1970). That, since crystalline rubredoxin can be chemically reduced and reoxidised by air without apparent damage to the crystal, no major conformational changes take place in the protein during the redox cycle was deduced by Lovenberg and Williams (1969). Technical problems have hindered progress of x-ray crystallographic studies on these proteins but some studies have been described by Gillard et al. (1965), Kraut, Strahs and Freer (1968) and Sieker and Jensen (1965). The most recent study and one in which resolution has been clearest is that on Cl. pasteurianum rubredoxin by Herriott et al.(1970). The electron density map at 2.5 Å resolution clearly shows that iron is coordinated to four sulphur atoms which occupy tetrahedral vertices. This is of especial interest since in this protein although there are 4 cysteine residues there is no "labile" sulphur to match the single iron atom in the molecule (Table III).

Adrenodoxin is reported (Dr.J.I.Mason -personal communication) to have been crystallised by Kimura et al. and Estabrook et al. but no details have been published as yet owing to difficulties with making x-ray crystallographic measurements.

Effects of x-irradiation on ferredoxin solutions: The studies of Krasnobajew (1971) suggest that the loss of the iron-sulphur chromophore and biological activity of Cl. pasteurianum ferredoxin following x-irradiation is caused by OH-radicals.

Molecular structure of iron-sulphur proteins

A considerable amount of attention has been focussed on this aspect of iron-sulphur protein chemistry in view of the interesting biological functions and properties of these enzymes. The structure of the polypeptide chain is becoming clear but the nature of the active centre and "labile sulphur" is still subject to controversy. This is due in part to there being different types of iron-sulphur

proteins such as ferredoxins, rubredoxins, HiPIP^{s} etc. as well as differences between members of each type. Some of the data to be discussed in this section is summarised in Table III.

Molecular weight: The early work on these proteins established the molecular weight of a number of them. Several reinvestigations have had to be made of the early figures and Table III shows currently accepted values. The general pattern which emerges is that rubredoxins have a molecular weight of about 6,000, bacterial ferredoxins about 6,000, plant and algal ferredoxins about 12,000, and the two HiPIP^{s} about 10,000. Chromatium ferredoxin is intermediate in molecular weight between clostridial ferredoxin and those of spinach, alfalfa and Scenedesmus (Matsubara, Jukes and Cantor, 1968); an intermediacy which is also reflected in its amino acid composition (Matsubara, 1968; Sasaki and Matsubara, 1967). Some of the Azotobacter proteins have comparatively high molecular weights of 20,000 and over (Shethna, 1970; Yoch et al., 1969).

For some years it was believed that adrenodoxin had a molecular weight of about 15,000-20,000 (Kimura and Suzuki, 1967). However its behaviour on gel-filtration in the course of the present work indicated that its molecular weight was more in keeping with values given for plant ferredoxins (i.e. about 12,000). A subsequent article by Kimura et al.(1969) confirmed the redetermination of the molecular weight of adrenodoxin by several methods to be about 12,000.

Iron and "labile" sulphide contents: A characteristic of all ISP^{s} is the presence of bound iron and (except in the rubredoxins) in most cases "labile" sulphur in equivalent quantities. The term "labile" or "inorganic" sulphur is used to denote that which is released in the form of hydrogen sulphide on acidification of a solution of an ISP. Table III shows the currently accepted values

for the iron, "labile" sulphur and cysteine residues of some ISP^S. A range of values is seen with clostridial ferredoxins having up to 8 atoms of Fe and 8 of "inorganic" sulphur while the majority of "plant ferredoxins" have two atoms Fe and rubredoxins no "inorganic" sulphur. The number of reducing equivalents transferred per protein molecule appears to bear little relationship to the number of Fe atoms present. Chemical and EPR methods showed that the iron in ferredoxins may be present in more than one valency state. Blomstrom et al. (1964) found at least 2 ferrous and 5 ferric iron atoms and Palmer et al. (1966) and Sobel and Lovenberg (1966) found equal amounts of ferrous and ferric forms in the native protein. Fry and San Pietro (1963) and Malkin and Rabinowitz (1967) have also made studies on spinach and clostridial ferredoxins but the question is still not resolved. It may well be that under the acid conditions of extraction of iron from the protein reduction may occur by the labile sulphur or sulphydryl groups.

The native state of the "labile" sulphur of ISP^S is not yet understood. It may be a form of "inorganic sulphur" as stated by Keresztes-Nagy and Margoliash (1966) and Malkin and Rabinowitz (1966b) or may arise by a β-elimination from polypeptide-bound cysteinyl residues in the presence of iron (Bayer, Parr and Kazmaier, 1965; Gersonde and Druskeit, 1968). These groups have obtained contradictory evidence as to the presence in the apoprotein of dehydroalanine, which should result from the cysteine β-elimination reaction. The problem has been studied by a variety of methods including EPR spectroscopy after exchange of "labile" sulphur with S^{33} (Beinert and Orme-Johnson, 1969; Tsibris et al., 1968) and Se^{77} and Se^{80} selenium (Gunsalus, 1968; Gunsalus et al., 1969).

Table III A comparison of certain structural and functional characteristics of some iron-sulphur proteins
(this data has been compiled from the "Principal references" and others cited in the text)

Type and source	Mol.wt.	Amino acid composition per molecule species, residues, Glx + Asx			Sulphur content per molecule labile, cysteine		Iron atoms per molecule	Reducing equivalents transferred per molecule	Redox potential (V)	Principal references
Ferredoxins										
1) spinach, parsley	11,500	17	97	26	2	5	2	1	-0.42	Arnon, 1969; Hall and Evans, 1969; Mayhew et al, 1969a.
2) Leucena glauca	11,500	17	96	26	2	5	2	1		Benson and Yasunobu, 1969.
3) cotton cotyledon	10,950	18	99	34	2	4	2			Newman, Ihle and Dure, 1969.
4) alfalfa	11,500	17	97	25	2	5-6	2	1		Keresztes-Nagy, Perini and Margoliash, 1969.
5) taro	12,000	17	98	26	2	5	2			Rao, 1969.
6) Scenedesmus	11,500	17	96	22	2	6	2	1		Matsubara, 1968.
7) Anacystis nidulans	11,000	16	105	26	1	6	2	1	-0.43	Yamanaka et al, 1969.
8) Chromatium	10,000	16	81	24	4	9	4	1	-0.49	Matsubara, Jukes and Cantor, 1968.
9) Chlorobium thiosulfatophilum	6,000	13	53-56	11	4-5	7	4-5			Buchanan, Matsubara and Evans, 1969.
0) M. aerogenes	(5,600)	11	54	12	7-8	8	7-8			Tsunoda, Yasunobu and Whiteley, 1968.
1) Peptostreptococcus elsdenii								2		Mayhew et al, 1969.
2) Desulfovibrio gigas	6,500	14	61	21	4	5	4			Laishley, Travis and Peck, 1969.
3) Cl. butyricum	(5,600)	11	55	14	7-8	8	7-8			Lovenberg, Buchanan and Rabinowitz, 1963
4) Cl. acidi-urici	6,000	11	55	12	8	8	8	2	-0.39	Rall, Bolinger and Cole, 1969.
5) Cl. pasteurianum	6,000	13	52-55	12	8	8	8	2	-0.39	Lovenberg, Buchanan and Rabinowitz, 1963; Tagawa and Arnon, 1968.

Rubredoxins										
1) Ps.oleovorans	12,800				0		2	2	-0.037	Peterson and Coon, 1968.
2) M.aerogenes	6,000	16	53	16	0	4	1			Bachmeyer et al, 1968a.
3) M.lactilycus	6,000	16	51	12	0	4	1			Lovenberg, 1966.
4) P.elsdenii	6,000	16	52	12	0	4	1			Mayhew and Peel, 1966; Newman and Postgate, 1968.
5) Desulfovibrio desulfuricans	6,500	15	60	15	0	4	1		-0.081 to + 0.011	Newman and Postgate, 1968.
6) D.gigas	6,500	16	61	13	0	4	1			Laishley, Travis and Peck, 1969.
7) Cl.pasteurianum	6,000	13	53	17	0	4	1	1	-0.057	Lovenberg and Sobel, 1965; Lovenberg and Williams, 1969.
HiPIPs										
1) Rhodopseudomonas gelatinosa	9,600	17	90	9	4	4	4		+0.33	Dus et al, 1967.
2) Chromatium	10,100	20	92	14	4	4	4	1	+0.35	Dus et al, 1967; Mayhew et al, 1969.
Other ISPs										
1) Cl.pasteurianum azoferredoxin	40,000				2		2			Jeng et al, 1969; Moustafa and Mortenson, 1969.
2) Azotobacter protein I	21,000	18	182	39	2	5-6	2	1		DerVartanian, Shethna and Beinert, 1969.
3) Azotobacter protein II	24,000				2		2	1		DerVartanian, Shethna and Beinert, 1969.
4) Azotobacter protein III	13-20,000	18	171	62	6-7	6	6-7			Shethna, 1970; Yoch et al, 1969.
5) Cl.pasteurianum hydrogenase	60,000				4		4			Nakos and Mortenson, 1971a.
6) synthetic ISP from BSA	69,000				6	34	6			Suzuki and Kimura, 1967.
"Hydroxylating" ISPs										
1) Putidaredoxin	12,500	18	114	26	2	6	2	1	-0.185 to -0.240	Gunsalus et al, 1969; Hall and Evans, 1969.
2) Adrenodoxin	12,000	17	94-115	24-30	2	4	2	1	-0.37	Kimura et al, 1969.

Petering, Fee and Palmer (1971) have shown that in adrenodoxin, putidaredoxin and several ferredoxins the inorganic sulphur can be oxidised to the zero valent oxidation state and covalently bound to the protein.

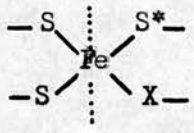
Active centre and iron-sulphur chromophore: The nature of the "labile" sulphur appears to be intimately bound up with that of the active centre and the chromophore since its liberation usually involves loss of biological activity and changes in the visible extinction spectrum. Villarejo and Westley (1963) also produced a substance with light extinction characteristics similar to ferredoxin by the interaction of sodium sulphide and cystine while more recently Suzuki and Kimura (1967) produced a synthetic "iron-sulphur protein" using bovine serum albumin. This preparation had extinction maxima at 279, 327, 386 and 630 nm and contained (bound) iron and acid-labile sulphur. The reaction medium contained 2-mercaptoethanol and ferrous ammonium sulphate. The work was extended by Lovenberg and McCarthy (1968) and McCarthy and Lovenberg (1968) who found that different, distinct synthetic ISP^S could be prepared from bovine serum albumin depending on the reaction conditions and even in the absence of 2-mercaptoethanol. Bayer, Krauss and Schretzmann (1969) produced a similar protein from ribonuclease and similar spectra by treating (ferric) haemerythrin with sulphide. Yang and Huennekens (1969, 1970) have produced a (protein-free) iron-mercaptoethanol-inorganic sulphide complex which has extinction maxima at 325, 412 and 450 nm and oxidises mercaptoethanol and reduces oxygen. The complex also shows EPR signals at $g = 4.1$, 2.01 and 1.96 at $110^{\circ}K$.

Similar experiments were carried out by Hong and Rabinowitz (1967), Jeng and Mortenson (1968) and Malkin and Rabinowitz (1966a) using clostridial apoferredoxins. They found that under controlled

conditions the inorganic iron and sulphur could be replaced into an apoprotein prepared by treatment with acid or the mercurial, mersalyl. The reconstituted protein had the spectral and enzymatic characteristics of the native enzyme. A similar reconstitution has been made with the iron-sulphur protein from Soya bean nodule bacteroids (Koch et al., 1970).

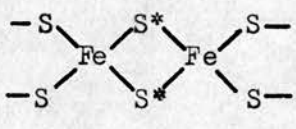
Controversy over many points does not permit as yet a unified theory as to the nature of the active site of "ferredoxins". Three theories of iron coordination structure have however been put forward -

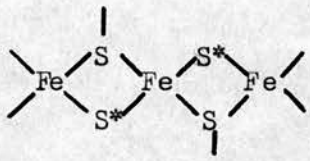
1) Cysteine ligand structure (Bayer, Parr and Kazmaier, 1965)



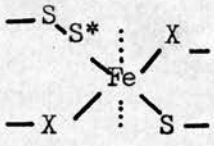
(S* represents "labile sulphur")

2) Sulphur bridged structure (Blomstrom et al., 1964; Brintzinger, Palmer and Sands, 1966; Tanaka et al., 1965)





3) Persulphide structure (Miller and Massey, 1965)



The iron-binding in rubredoxin, which is unusual in the absence of "labile" sulphur, is discussed by Eaton and Lovenberg (1970) and Lovenberg (1966). The iron bonding to the mercaptide sulphurs of the only four cysteinyl residues of rubredoxin is roughly tetrahedral (Eaton and Lovenberg, 1970). Further discussion of the iron-sulphur chromophore is also found in Bearden et al. (1965), Gibson et al. (1966), Kimura (1968) and Palmer et al. (1966).

Investigations on the iron-sulphur chromophore and iron valency state of adrenodoxin have been made (Kimura 1968). The results obtained did not support the theory that "labile" sulphur arose from cysteinyl residues. Furthermore α-α'-dipyridyl-treated and reisolated apoadrenodoxin regained its iron-sulphur chromophore and enzymic properties after treatment with ferrous salt, 2-mercaptoethanol and Na_2S.

Amino acid composition and sequencing studies: The amino acid composition of a variety of ferredoxins shows a high content of acidic residues (Table III) and a low content of basic amino acids in a single polypeptide chain (Benson, Mower and Yasunobu, 1966; Keresztes-Nagy and Margoliash, 1966; Lovenberg, Buchanan and Rabinowitz, 1963; Matsubara and Sasaki, 1968; Tanaka et al., 1964, 1966). The amino acid composition of putidaredoxin is similar (Gunsalus et al., 1969). Hydrogenase, azoferredoxin and molybdoferredoxin contain 2, 2 and 3 subunits respectively (Nakos and Mortenson, 1971a,b,c). Clostridial ferredoxins lack histidine, arginine, methionine, leucine and tryptophan (Lovenberg, Buchanan and Rabinowitz, 1963; Tanaka et al., 1964, 1966) and the contents of these amino acids were found to be low in spinach and alfalfa ferredoxins as well (Matsubara and Sasaki, 1968). Clostridial rubredoxin is probably the only known constitutive cell protein to have N-formylmethionine as its N-terminus (McCarthy and Lovenberg, 1970). Clostridial ferredoxins have a repeating sequence of 22-26 residues about a central phenylalanine (Tanaka et al., 1966), but statistical tests are said not to bear out the hypothesis that each half derives from the repetition of a smaller peptide (Urbain, 1969). Although the probable non-helical structure of ferredoxins has been suggested (Tanaka et al., 1966), the possibility of some α-helix and some

β-helix in ISP^s has also been proposed (Keresztes-Nagy, Perini and Margoliash, 1969; Kimura et al., 1969).

Kimura et al. (1969) have suggested differences between adrenodoxin and spinach ferredoxin in the possible presence of α-helix in the former and β-helix in the latter. Their proposed amino acid composition of bovine adrenodoxin (Kimura, 1968; Kimura et al., 1969) shows many features similar to that of other ISP^s but differs to some extent from that of adrenodoxin published shortly afterwards by Tanaka, Haniu and Yasunobu (1970). The latter work also gives the primary sequence of bovine adrenodoxin and suggests which of the five cysteine residues may be involved in iron binding.

Primary and iron-sulphur chromophore structures published for similar proteins include those for Micrococcus rubredoxin (Bachmeyer et al., 1967, 1968a; Bachmeyer, Yasunobu and Whiteley, 1967, 1968), Peptostreptococcus rubredoxin (Bachmeyer et al., 1968b), Clostridium ferredoxin (Benson, Mower and Yasunobu, 1966, 1967; Rall, Bolinger and Cole, 1969) and Clostridium rubredoxin (Eaton and Lovenberg, 1970). Fig. 12 shows some proposed structures.

Genetic, isoenzyme and evolutionary studies

These aspects which are so different in themselves are treated together since in the ferredoxins, examination of these points entails comparison of the amino acid sequences of different proteins.

Multiple forms of ferredoxins have been suspected to occur in spinach (Matsubara and Sasaki, 1968; Matsubara, Sasaki and Chain, 1967) and alfalfa (Keresztes-Nagy, Perini and Margoliash, 1969) ferredoxins. Benson and Yasunobu (1969a,b) have pursued the investigation of genetic aspects and identification of isoenzymes by comparison of sequences of ferredoxin isolated from the leaves of the tree, Leucena glauca. Their studies indicate heterogeneity of

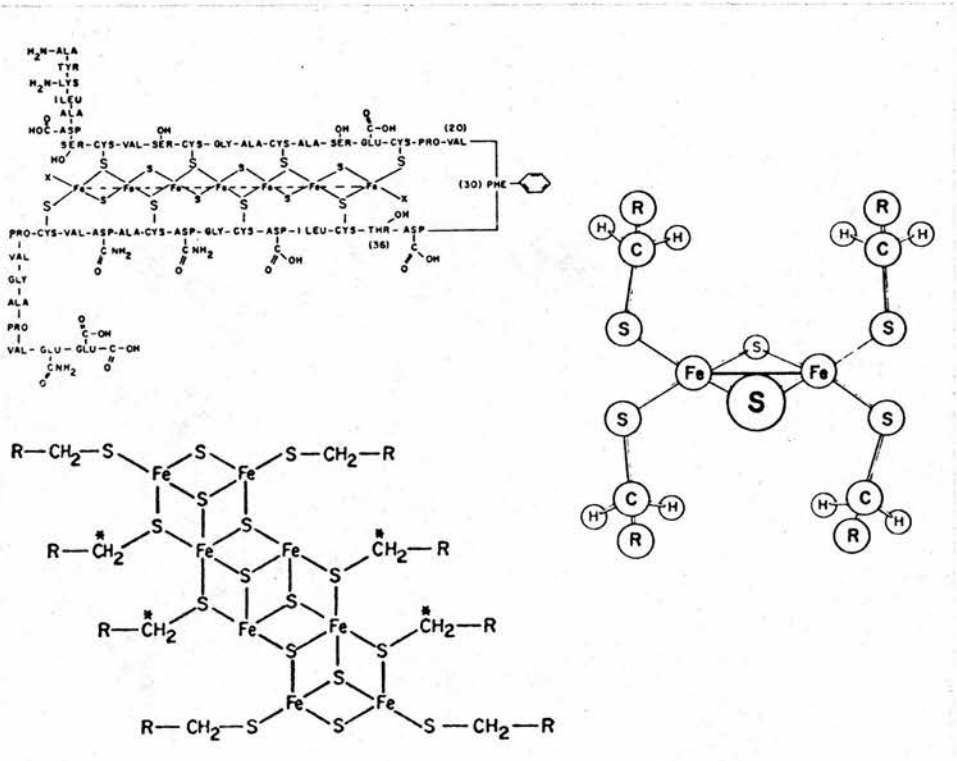


Fig. 12 Proposed structures of "iron-sulphur proteins"

Above left - Cl. pasteurianum ferredoxin (from Tanaka et al, 1965)

Centre right - Model for the iron-sulphur centre in spinach and parsley ferredoxin (from Poe et al, 1971b)

Below left - Model for iron-sulphur complex of Cl. pasteurianum ferredoxin (from Poe et al, 1970)

sequences caused by different genes rather than ambiguity in translation of the genetic code and further indicate the possible presence of as many as eight forms of the protein.

The considerable work accomplished on the sequencing of iron-sulphur proteins and the pattern which began to emerge have inevitably led to consideration as to the possible presence of an evolutionary trend. A considerable amount of data has been amassed from the laboratories of Matsubara and of Yasunobu in support of this possibility. It has been suggested that (a) the iron-sulphur proteins are possibly more ancient than the cytochromes (Benson and Yasunobu, 1969a; Margoliash and Fitch, 1967), (b) gene duplication occurred in the evolution of non-photosynthetic bacterial ferredoxins (Eck and Dayhoff, 1966) and (c) spinach and bacterial ferredoxins evolved from a common ancestor rather than there being a direct evolutionary progression. Fig. 13 shows a phylogenetic tree of the ferredoxins (Matsubara, Jukes and Cantor, 1968). Discussion of the evolutionary relationships of various iron-sulphur proteins may be found in Benson, Mower and Yasunobu (1967), Buchanan, Matsubara and Evans (1969), Matsubara, Jukes and Cantor (1968), Tsunoda, Yasunobu and Whiteley, (1968), Urbain (1969) and Weinstein (1969).

Conclusion

The above account attempts to give an idea of the interesting and diverse properties of some of those proteins which are commonly called the "iron-sulphur proteins" or the "electron-transferring non-haem iron proteins". Much more data will be needed before it is possible to classify them satisfactorily though many of the more studied members can now be placed in one of the following categories: Chloroplast ferredoxin, photosynthetic bacterial ferredoxin, non-photosynthetic bacterial ferredoxin, rubredoxin and HiPIP. The first

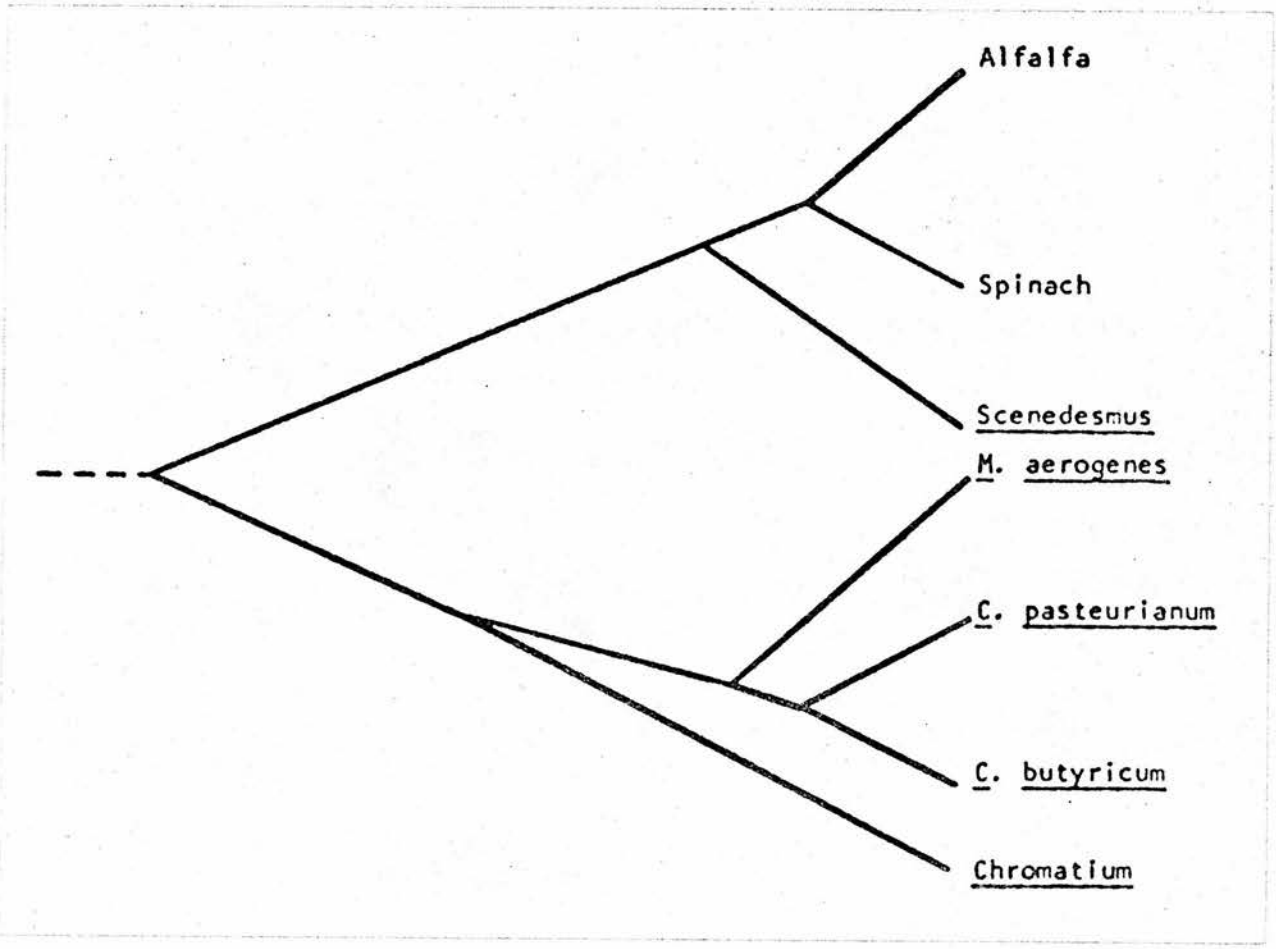


Fig. 13 A phylogenetic tree of ferredoxins

(from Matsubara, Jukes and Cantor, 1968)

three categories have been termed ferredoxins of Type 1, Type 2 and Type 3 respectively (Matsubara, Jukes and Cantor, 1968) and adrenodoxin has characteristics most similar to the chloroplast or Type 1 ferredoxin.

G. Cytochrome P450

Omura and Sato (1964a,b) in following up early indications of other workers concluded after extensive investigations that a b-type cytochrome was involved in hepatic microsomal mixed-function oxidations. This protein which was termed cytochrome P450 has since been identified as a component of a variety of mixed-function oxidases and in those cases is thought to be the enzyme on which the site of hydroxylation of substrate is located (see Figs. 6 and 7). Whereas the only known function of cytochrome P450 is in mixed-function oxidations it is thought not to participate in some of them such as the ω-hydroxylation of alkanes by Pseudomonas oleovorans (Peterson and Coon, 1968) or the 4-methylsterol demethylations by hepatic microsomes (Gaylor and Mason, 1968). A bacterial demethylation may however be cytochrome P450-dependent (Broadbent and Cartwright, 1971) as are microbial n-octane oxidation and (camphor) 5-exo-methylene hydroxylation (Cardini and Jurtshuk, 1968; Dus et al., 1970).

The participation (or not) of cytochrome P450 in a given mixed-function oxidation is often decided by comparison of the known characteristics of cytochrome P450 and the characteristics of the reaction being examined. Thus cytochrome P450 reduced biologically, e.g. with NADPH and cytochrome P450 reductase, or chemically, e.g. with dithionite, combines with carbon monoxide, CO, to form a pigment with a spectral extinction at 450 nm. Cytochrome P450-dependent hydroxylations are correspondingly inhibited by carbon monoxide. Both reduced cytochrome P450-CO complex dissociation and reversal of carbon monoxide inhibition of enzyme activity are effected by light

of 450 nm wavelength. Correlation may also be made between the presence of cytochrome P450 in a tissue and the hydroxylation studied. However Gaylor and Mason (1968) claim that hepatic microsomal sterol demethylation is not cytochrome P450-dependent notwithstanding the presence of cytochrome P450 in the tissue. Similar observations were made by Matthijssen and Mandel (1970) regarding steroid 21-hydroxylation by adrenocortical microsomes, while Mason and Boyd (1971) found active steroid hydroxylation by placental mitochondrial protein preparations containing little demonstrable cytochrome P450. It would appear that in many instances the "concentration"of cytochrome P450 is not the factor which limits the rate of a hydroxylation. Cytochrome P450 is readily converted to an inactive form of which the reduced-CO complex has an extinction maximum not at 450 nm but at 420 nm (Omura and Sato, 1964a,b). This inactive "cytochrome P420" can however in certain states combine with substrates to give difference spectra usually seen only with cytochrome P450 preparations (Shoeman, White and Mannering, 1969).

The difference spectra produced by substrates are a comparison of the spectrum of cytochrome P450 modified by substrate with that of unmodified cytochrome P450. Such spectra have been recorded for a variety of substrates (see Gillette et al, 1969) and have been divided into Type I spectra which have a peak of extinction at 390 nm and a trough at 420 nm (as with hexobarbital and certain other drugs) and Type II spectra which have a trough at about 393 nm and a peak at about 430 nm (as with aniline and certain other basic amines). The ability of a given preparation of cytochrome P450 to form a difference spectrum with a "substrate" is not however necessarily indicative of an ability of that species of cytochrome P450 to effect the hydroxylation of that "substrate" (Ichikawa, Kuroda and Yamano,

1970). The same authors report that the cytochrome P450 content of the mitochondria and microsomes of the zona fasciculata of the mammalian adrenal cortex is higher than in the zona reticularis and zona glomerulosa. Leibman, Hildebrandt and Estabrook (1969) and Schenkman et al.(1969) are two of the many groups who have investigated the possibility that more than one species of cytochrome P450 exists in hepatic microsomes in view of the diversity of substrates hydroxylated in this tissue. Although they concluded that only one cytochrome P450 protein species exists in hepatic microsomes, Jefcoate, Hume and Boyd (1970) succeeded in separating adrenal mitochondrial cytochrome P450 into two fractions specific for cholesterol side-chain cleavage and 11 β-hydroxylation of deoxycorticosterone. These authors also found that the 11 β-hydroxylase activity was correlated with a low-spin form and side-chain cleavage activity with a high-spin form of cytochrome P450. Detailed EPR studies of the high-spin and low-spin forms of cytochrome P450 from liver microsomes and Pseudomonas putida have been recently reported (Peisach and Blumberg, 1970; Tsai et al, 1970). The latter enzyme is soluble while the hepatic microsomal and adrenal microsomal forms are firmly membrane bound. The adrenal mitochondrial cytochrome P450 is essentially located in the inner membrane (Satre, Vignais and Idelman, 1969).

While problems of solubilization delayed progress on the purification of mammalian cytochrome P450, the Pseudomonas (camphor-hydroxylating) cytochrome P450 has been purified and crystallised (Yu and Gunsalus, 1970). Its chemical characterisation shows the presence of a single molecule of ferriprotoporphyrin IX and a small carbohydrate moiety and that it is an acidic protein (Dus et al, 1970). The formation of this enzyme is known to be induced by growth of the cells on camphor as sole carbon source (Gunsalus, 1968) while the synthesis of the hepatic microsomal enzyme is

known to be induced by a variety of agents (see Gillette et al., 1969).

H. Aims of this investigation

At the commencement of the investigation reported here it was known that the hydroxylation of steroids in the adrenal cortex mitochondria was effected by a complex of at least three distinct proteins (Nakamura, Otsuka and Tamaoki, 1966; Omura et al, 1966). Both in this tissue as well as in others the cytochrome P450 component had attracted a great deal of interest and considerable investigation had been made of its unique structural and enzymatic characteristics. However, at that time comparatively little information had been reported on the properties of adrenodoxin and even today the information published on the flavoprotein, adrenodoxin reductase, is scanty. Since processes relating to the mechanism of oxygen-insertion into the substrate molecule were already being thoroughly investigated it was decided to attempt to purify the components of cytochrome P450 reductase, investigate their properties and attempt to establish the stoichiometry of the reconstitution of the adrenal mitochondrial hydroxylase - which had not been reported at that time. The possible presence of separate cytochrome P450 reductases corresponding to separate steroid-hydroxylating activities and postulated multiple cytochrome P450 moieties could also be investigated. Factors affecting the production of reduced NADP in the adrenal cortex could be investigated, as well as those influencing the supply of electrons to the locus of steroid hydroxylation. Finally the absence of an adrenodoxin-like protein in adrenal microsomal and hepatic steroid hydroxylating enzyme systems had also been noted and this provoked the query as to the presence of adrenodoxin in adrenal mitochondrial systems.

CHAPTER 2

METHODS

Preparation of bulk quantities of mitochondria

At the time the study was commenced, experimentation commonly carried out in this laboratory was with small quantities of mitochondria or enzyme extracts prepared from a few freshly collected bovine adrenal glands. Since in protein purification the work involved in many steps of the fractionation process is of the same order whether a large or a small quantity of enzyme is being handled, it was decided to scale-up the procedure for preparing mitochondria. Kimura (1968) has described a method of isolating adrenodoxin by absorption onto DEAE-cellulose from unfractionated adrenal homogenate. The present study being devoted however to the examination of the (mitochondrial) enzymes concerned in cholesterol side-chain cleavage and DOC 11β-hydroxylation, the initial preparation of mitochondria was an essential step in the purification procedure. The preparation of adrenocortical mitochondria differs from those from other soft tissues such as liver, mainly in the need to remove the inner adrenal medulla and the outer capsule. Initial attempts to mince the capsule and cortex with scissors or a blade were not very successful as the fibrous fragments of capsule tended to hinder homogenisations at a subsequent stage. A satisfactory method presently followed is described below (see Fig. 14).

Between 40-100 (commonly about 70) bovine adrenal glands were freshly collected from the Edinburgh slaughter house shortly after removal from the animals which were mainly Aberdeen-Angus crosses or Hereford crosses, but variable as to sex and age. The glands were transported on ice to the laboratory and processed at 0-4^{o}C without delay due to the need to isolate as many intact mitochondria as possible and the possible lability of the enzymes at this stage. The glands were defatted with scissors and then split down the middle.

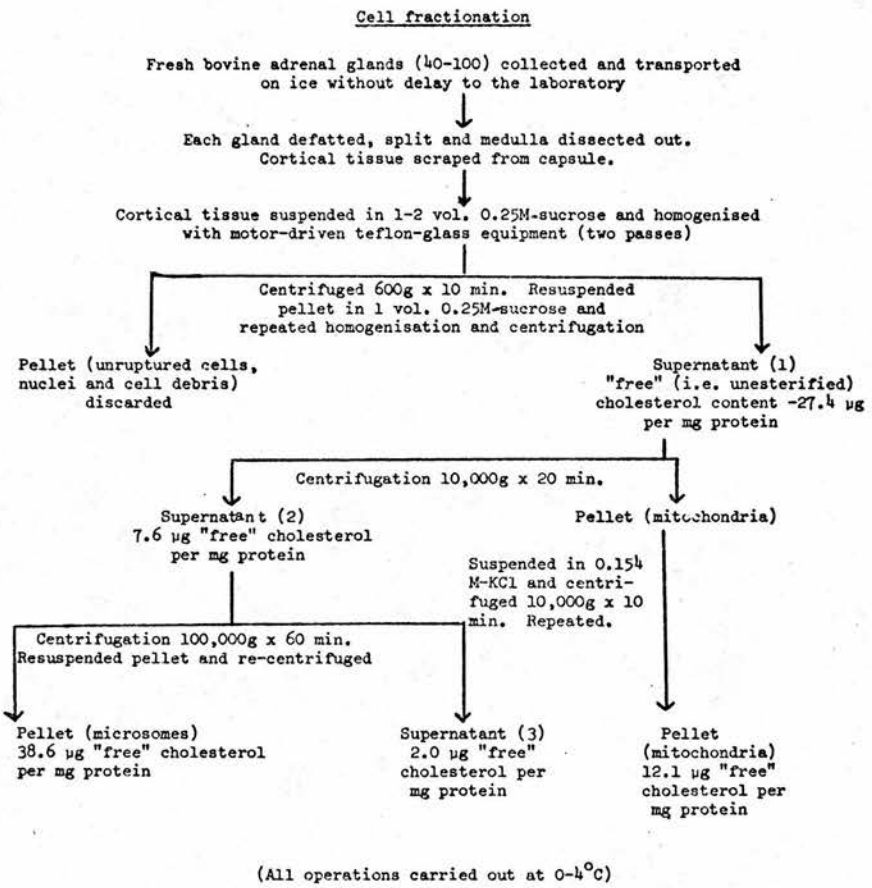


Fig. 14 Technique for preparation of adrenocortical cell subfractions

The medulla was dissected out from each half and the cortex next scraped from the capsule with a scalpel. (Averages taken over a year's material showed that 100 fresh bovine adrenals yielded 998 g defatted glands or 510 g scraped cortical tissue.) The scraped tissue was suspended in 1-2 vol. of cold 0.25 M-sucrose and homogenised in a motor-driver teflon-glass equipment designed to produce mitochondria. Two passes of the homogeniser were usually adequate and if a large amount of fibrous capsular material was observed in any particular preparation the whole was passed through a single layer of muslin, the material retained being rinsed with cold sucrose into the filtrate. A suitable alternative to the motor-driven homogeniser was a Potter-Elvehjem type hand homogeniser. The homogenates were combined and centrifuged (MSE 17,000) at 600 g for 10 min to sediment nuclei, cell debris and unbroken cells. The supernatant was retained, the pellet being resuspended in 1 vol. sucrose by gentle hand-homogenisation and recentrifuged. The resulting pellet was rejected and the supernatants combined and centrifuged at 10,000 g for 20 min. (The pellet now formed was of the mitochondria, but a visually similar material appeared to float at the surface when the supernatant was being decanted. Acetone powders were prepared of the "true" mitochondria and of the floating material and the cholesterol side-chain cleavage activity tested. Under similar conditions, "true" mitochondria gave 6.9% conversion/mg protein/60 min and the "floating material" gave 5.08%/mg protein/60 min. The floating material may have been light or partially lysed mitochondria.) The pellet was washed twice in cold 0.154 M-KCl; being recovered by centrifugation for 10 min at 10,000 g. The success of the method as a means of preparing intact mitochondria was judged by electron microscope examination following fixation in Palade's Osmic acid, staining and

embedding in Araldite. Fig. 15 shows a field (x 32,000) from such an electron micrograph and validates the method described above for preparing large quantities of mitochondria. (1,000 g of scraped cortical tissue commonly yielded about 260 g of fresh mitochondria free of excess buffer.)

Preparation of acetone powders and lyophilised material

Fresh mitochondria could be used as such in experiments or disrupted by sonication and used for the extraction of particular enzymes immediately. Often however mitochondria were stored in the deep-freeze (-20°C) for use as required as acetone powders or lyophilised material. Each process had certain advantages and the choice made depended on the intended use of the mitochondrial preparation. Lyophilisation was less liable to misadventure being a less rigorous treatment and required fewer manipulations in the form of redistillation of acetone and the lengthy acetone- and ether-washing of the powder. However preparation of an acetone powder was helpful in that it tended to remove a large proportion of the endogenous cholesterol present in adrenal mitochondria. High levels of this endogenous cholesterol tend to dilute the [4-^{14}C] radioactive cholesterol tracer used in side-chain cleavage determinations. This effect could be minimised by increasing the enzyme input or lengthening the time of incubation, thus increasing the conversion of [4-^{14}C] cholesterol. However this results in undesirably high levels of "cold" pregnenolone building up and the secondary effects of unduly incubating the enzyme at 37°C; both of which factors would tend to mask the true initial rate of cholesterol side-chain cleavage.

Lyophilisation was carried out by the standard method of applying the mitochondria in water as a smooth paste to a round-bottomed flask, freezing this as a shell with solid carbon dioxide and ethanol and freeze-drying overnight.

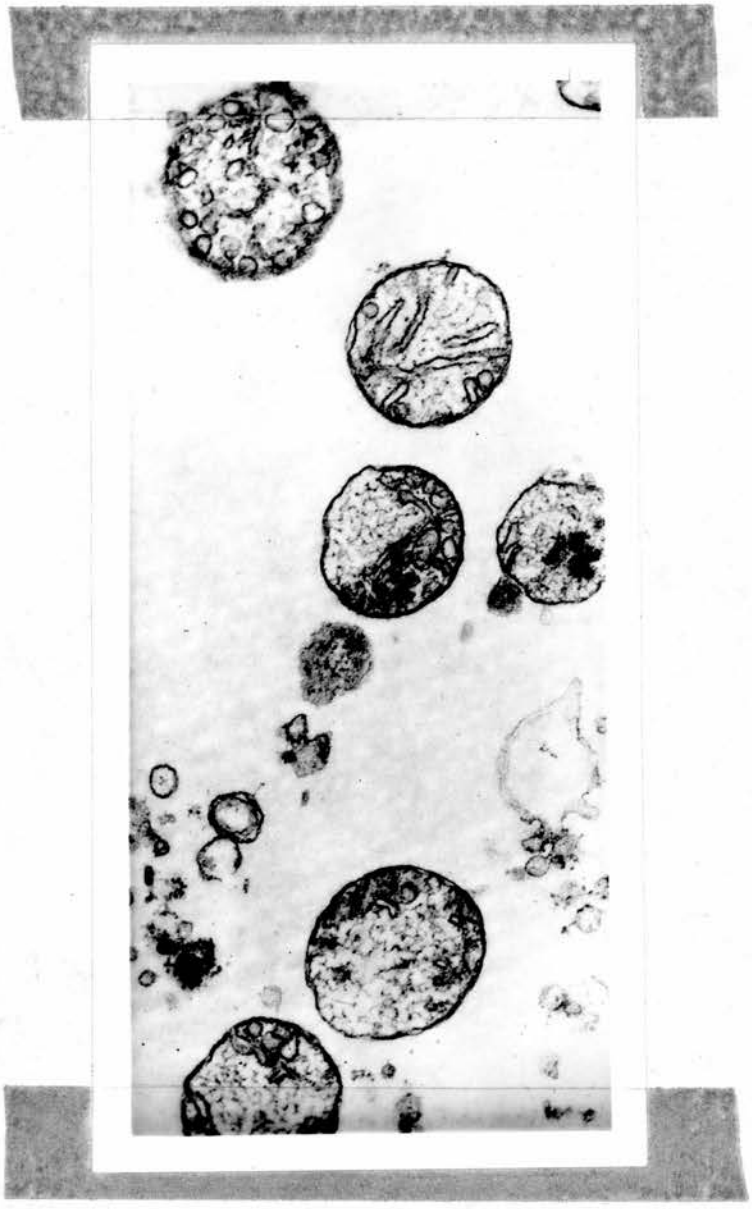


Fig. 15 Adrenocortical mitochondria (x 32,000)

Method of isolation as described in Fig. 14. Stained with uranyl acetate and lead citrate after fixation in Palade's osmic acid.

Acetone powders were prepared by pouring a thin paste of mitochondria (after a brief sonication) very slowly into 20 volumes of redistilled acetone cooled in solid carbon dioxide. This mixture in a glass beaker was stirred rapidly with a glass rod to ensure rapid contact of the solid material with the cold acetone. The resultant dehydrated protein was filtered off onto a filter paper in a precooled Buchner funnel, and washed with several volumes of cold redistilled acetone. Great care was needed not to suck the protein dry of solvent either at this stage or the next, which was the wash with ether precooled in solid carbon dioxide. After the ether wash, the protein (and filter paper) were transferred in a suitable container rapidly to a desiccator and placed under vacuum. After an hour, the vacuum was released and the still slightly moist protein removed from the filter paper and ground in a mortar. The whole preparation was returned to the desiccator and left under vacuum overnight in the cold. 1 mg acetone powder protein contains 0.61 µg of residual cholesterol while supernatants (100,000 g x 30 min) resulting from solubilisation of these powders contain 2.5 µg cholesterol/mg protein and pellets 0.35 µg cholesterol/mg protein.

The dry powders resulting from lyophilisation or acetone treatment could be stored in the deep-freeze for months without detectable deterioration. (1,000 g of scraped cortical tissue gives about 24.7 g lyophilised mitochondria, 32.8 g acetone powder or 18 g resuspended protein. These figures indicate actual recovery rates on different preparations.)

Buffers

For the preparation of mitochondria 0.25 M-sucrose and 0.154 M-potassium chloride solutions were used. For the regulation of pH Tris-HCl buffer pH 7.4 was used except where otherwise indicated.

The buffer pH values stated were those at the time of preparation at room temperature. Tris-HCl buffer does vary in pH with temperature and affect biuret determinations but was used since phosphate buffer is reported to harm adrenodoxin (Omura et al.,1965a; Omura et al., 1967). Adjustments were made to correct for any important discrepancies. All other buffers used were made according to Gomori (1955).

Assay of cholesterol side-chain cleavage (CSCC)

The measurement of cholesterol side-chain cleavage was carried out by a method used successfully in this laboratory over several years by Mason and Boyd (1970), Simpson and Boyd (1966), Sulimovici and Boyd (1968a). Typical incubation mixtures contained ~~0.05 ml~~ $NADP^+$ (0.5 µmol), glucose-6-phosphate dehydrogenase (0.5 units), glucose-6-phosphate (40 µmol), [4-^{14}C] cholesterol (2.5 nmol, 125 nCi) in acetone (50 µl), enzyme preparation and Tris-HCl buffer (0.5 mmol, pH 7.4). The total volume of the reaction mixture was 5 ml.

~~7.4 to 5 ml.~~ The [4-^{14}C] cholesterol was cleaned by TLC on silica-gel H-coated plates every two weeks or just before use. The enzyme preparation was in Tris-HCl 0.1 M, pH 7.4 and dialysed against the same buffer before use and the other water-soluble ingredients were also dissolved in buffer except for glucose-6-phosphate dehydrogenase where the concentrated stock solution was diluted with distilled water. Where several incubations were carried out the [4-^{14}C] cholesterol was added to and allowed to equilibrate with the enzyme extract before dividing the latter into the various incubation flasks. The reaction was initiated by the addition of the NADPH generating mixture (freshly mixed and kept in ice) which included the additional buffer. The incubations were carried out with agitation for 5 or 10 min in a Dubnoff incubator at 37°C.

The radioactive products of the reaction were extracted and separated by thin-layer chromatography on silica-gel H-coated plates

(solvents - diisopropyl ether:petroleum ether (60-80°C): acetic acid::70:30:2 - 2 runs) and the rate of cholesterol side-chain cleavage determined as a percentage of [4-^{14}C] cholesterol converted to [4-^{14}C] pregnenolone and [4-^{14}C] progesterone (Mason, 1970; Simpson and Boyd, 1966; Sulimovici, 1968). The percentage conversion of radioactive cholesterol to radioactive pregnenolone and radioactive progesterone in a given series of incubations is a measure of the enzyme activity under the different conditions.

Assay of 11β-hydroxylation of 11-deoxycorticosterone (DOC)

The 11β-hydroxylation of DOC was followed by means of incubations as for cholesterol side-chain cleavage with the difference that DOC (0.5 μmol) in ethanol (10 μl) was used instead of radioactive cholesterol. Between two and three ml of the completed reaction was extracted with 15 ml dichloromethane. Ten ml of the dichloromethane extract was added to 2.5 ml of 30% ethanol in concentrated sulphuric acid, mixed thoroughly and allowed to stand. The upper layer was discarded and the fluorescence of the acid extract read with excitation at 470 nm and emission at 530 nm (Mattingly, 1962; Mattingly et al, 1964). The method was found to give reproducible results when readings were taken 45 min after addition of the ethanol and sulphuric acid reagent. Quantitation of conversions were made by including specimens containing known amounts of corticosterone and have been expressed in terms of percentage conversion. This provides a measure of the enzyme activity under different conditions.

~~Assay of protein and nitrogen contents of proteins~~

~~Assays of protein content were routinely made using the biuret (Gornall, Bardawill and David, 1949) or Lowry (Lowry et al, 1951) methods. Precautions were taken in using the biuret reaction in the~~

Enzymatic assay of the flavoprotein

The assay of the flavoprotein was carried out by measuring the reduction of dichlorophenolindophenol (DCPIP) spectrophotometrically at 590 nm.The reaction cuvette contained DCPIP (0.1 μmol),$NADP^+$ (0.5 μmol),glucose-6-phosphate dehydrogenase (0.5 units),glucose-6-phosphate (40 μmol),FAD (3 nmol),enzyme preparation and Tris-HCl buffer (0.3 mmol,pH 7.4) in a final volume of 3 ml.All reagents were dissolved in buffer and the reaction started by the addition of flavoprotein to the sample cuvette.The slow nonenzymatic reduction of DCPIP by NADPH and reoxidation by air were compensated for by having these reagents in the reference cuvette and replacing the flavoprotein component with buffer.The assay was adapted from that described by Omura et al.(1966) significantly in the lower DCPIP concentration and the inclusion of FAD.These adaptations significantly enhanced the reaction velocity.The millimolar extinction coefficient ($E_{mM}^{590\ nm}$) of DCPIP of 19 was used (Savage,1957).

The measurement of DCPIP reduction as an assay for adrenodoxin reductase is inadequate in several respects.It is non-specific since DCPIP is a known substrate for other flavoproteins (see Slater,1966), inorganic salt concentrations can affect hydrogen ion transfer to DCPIP (Webb,1963 p.835),FAD and DCPIP interact with each other directly (Yagi,Ozawa and Okada,1959) as well as with the enzyme and DCPIP interacts with NADPH generator especially in concentrated solution. Omura et al.(1966) have already commented on the harmful effect of preincubating the flavoprotein with DCPIP.The method is still the most convenient for monitoring the progress of a purification but checks need to be carried out periodically to ensure that the flavoprotein being isolated is adrenodoxin reductase.The checks which are available are the adrenodoxin-dependent NADPH-cytochrome c reduction activity (Omura et al.,1966),the reduction of the adrenodoxin chromophore in substrate quantities (Kimura and Suzuki,1967), the

reduction of cytochrome P450 in substrate quantities (Omura et al., 1966) and the reconstitution of the steroid hydroxylating system (Omura et al.,1966).The first is the most convenient, being rapid and economical of purified adrenodoxin and cytochrome P450 enzyme preparations and was routinely used.The requirement of both the flavoprotein and adrenodoxin preparations for NADPH-cytochrome c reduction was verified initially.

Assay of protein and nitrogen contents of proteins

Assays of protein content were routinely made using the biuret (Gornall,Bardawill and David,1949) or Lowry (Lowry et al.,1951) methods.Precautions were taken in using the biuret reaction in the

(continued on next page-

presence of sucrose and Tris buffer since these interfere with the colour developed (Robson, Goll and Temple, 1968). Similar corrections were made for Tris interference in the Lowry method of estimation. The method of protein measurement of purified adrenodoxin reductase is described in Chapter 3 and of purified adrenodoxin in Chapter 4.

The estimations of nitrogen contents were carried out on an automatic analyser using hypochlorite and phenol reagent after the protein solution had been digested in a sulphuric and perchloric acid mixture containing hydrogen peroxide (the method was found to be accurate to ± 1%).

Preparation of cytochrome P450

Adrenal cytochrome P450 was prepared by the method of Jefcoate, Hume and Boyd (1970) although separation of the cholesterol side-chain cleavage and DOC 11β-hydroxylation components were not necessary for most experiments.

A protein preparation containing cytochrome P450 and adrenodoxin reductase was prepared by sonicating mitochondria and washing the fragments repeatedly by suspension in 0.1 M-Tris-HCl pH 7.4 and centrifugation. This preparation could only hydroxylate steroids, after the washing procedure, when adrenodoxin was added. ~~A specimen in 50mM-~~ Cytochrome P450 concentration decreased from 30.8 μM to 24.7 μM after 24 hour storage at 4°C in 50mM-Tris buffer. Cytochrome P420 increased in proportion. ~~correspondingly.~~

Assay of cytochrome P450

The assay of cytochrome P450 was carried out by the CO-reduced extinction at 450 nm as described by Omura and Sato (1964b). An extinction at 420 nm (due to the cytochrome P420) was only taken into account for intramitochondrial localisation of the haemprotein.

Estimation of cholesterol

(a) Liebermann-Burchard method

The above method as modified and described by Abell et al.(1952) and Boyd (1964) was found to give reproducible results in the determination of cholesterol. This was extracted from protein with alcoholic KOH and petroleum ether.

(b) Gas-liquid chromatography (GLC)

The cholesterol contents of subcellular fractions were determined after thoroughly extracting aliquots(1 ml) of each protein suspension with ethanol. The ethanol was extracted with 9 volumes of chloroform. High specificity [7α-^{3}H] cholesterol (50,000 cpm) was added to the chloroform extract which was then dried down by gentle warming under a stream of nitrogen gas. The residue was taken up in a little chloroform which was used to wash down traces from the sides of the container and transferred quantitatively to a silica-gel H-thin-layer chromatography plate. The plate was developed in diisopropyl ether:petroleum ether (60-80°C):acetic acid::70:30:2 (two runs) and the cholesterol peak recovered quantitatively in acetone. The sample was dried down and redissolved in a known quantity of redistilled acetone. The recovery of cholesterol was estimated by counting the radioactivity of one tenth of the sample in a Packard Tricarb liquid scintillation spectrometer (see Mason, 1970). The estimations of these cholesterol solutions was performed on a Pye model 104 chromatograph unit using a calibration curve determined immediately before and checked frequently in the course of a series of experimental values. The calibration curves were prepared at various sensitivity settings using standard solutions of cholesterol in acetone. The various values for nonesterified cholesterol were related to and expressed relative to the protein content of the subcellular fractions from which they were derived (Fig. 14).

Estimation of "acid-extractable" and "acid-nonextractable" iron in proteins

The "acid-extractable" and "acid-nonextractable" contents of iron in a protein preparation give a measure of the "non-haem" and "haem"

iron present respectively. Reliable results were obtained by the protocol laid out in Fig.16 using the sensitive reagent bathophenanthroline sulphonate (4, 7-diphenyl-1, 10-phenanthroline sulphonate). Iron from haemproteins was extracted using hydrogen peroxide and perchloric acid and boiling (see Mason, 1970). Acid-washed glassware was used to eliminate contamination. While good linearity was obtained in the standard curve it was found in the course of this work that doubling the input of bathophenanthroline sulphonate to 0.2 mg per assay increased the working maximum from 20 to 40 ng-atom Fe^{++}. The method is an adaptation from Adler and George (1965), Massey (1957) and Ramsay (1958).

Electrophoresis of purified protein preparations

The purity of preparations of adrenodoxin and adrenodoxin reductase were examined using electrophoresis as a criterion. Early investigations were made using strips of cellulose acetate (Oxoid) and staining with Ponceau S in 6% sulphosalicylic acid.

An attempt was made to devise a stain which would show up the position of the non-haem iron protein on cellulose acetate after electrophoresis. Samples of adrenodoxin were electrophoresed on adjacent strips. One was stained with Ponceau S to locate the protein. The other was immersed in pyridine containing 10% ascorbic acid solution (5 mg/ml). The strip was then placed in a cleaned and dry dish and bathophenanthroline sulphonate solution (2 mg/ml) dripped on using a cleaned Pasteur pipette. This was followed by a little glacial acetic acid. A pink colour developed in about three minutes in the same region as protein was found on other strips. The intensity of the colour was not stable nor was the definition of the bands very sharp (probably due to partial solubilisation of the iron by the acid) but the method is useful in locating a non-haem iron protein among several protein bands. The stain is applicable also to disc electrophoregrams.

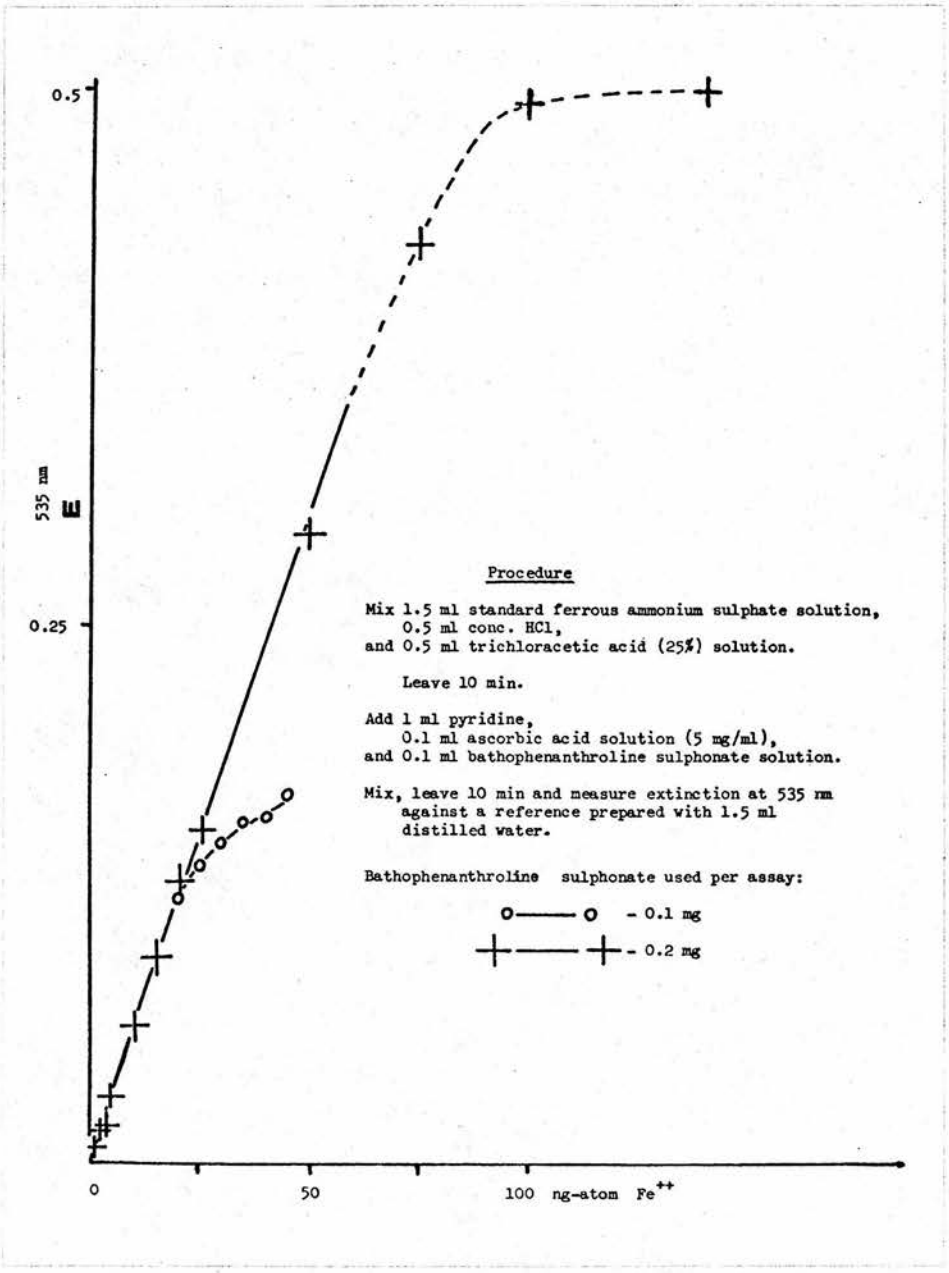


Fig. 16 The method for the determination of ferrous iron in solution

The inclusion of the higher level of bathophenanthroline sulphonate increased the range of this assay.

The disc-gel was soaked overnight in bathophenanthroline sulphonate (2 mg/ml) solution with dithionite as a reducing agent. It was then removed from the bathophenanthroline solution and acidified with glacial acetic acid when a pink colour developed corresponding to the adrenodoxin.

Separation of protein bands following disc-gel electrophoresis was sharper than that obtained by electrophoresis on strips of cellulose acetate. The 10% polyacrylamide gel was prepared and the protein was stained as described by Cruft (1962). A clear band definition was obtained.

Chromatography and determination of phospholipids

Separation of phospholipids was carried out by TLC on silica-gel G in chloroform:methanol:water::65:25:4. The spots were visualised by dipping the TLC plates in iodine vapour. Confirmation that the spots were due to phospholipid was obtained by running duplicate TLC plates in petroleum ether (40-60°C):diethyl ether:acetic acid::80:20:1 when phospholipids remain at the origin (Chahl and Kratzing, 1970). Records were made by photography or spraying the plates with 50% sulphuric acid and heating.

Quantitation of phospholipids (or the phospholipid content of proteolipids) was carried out by the determination of the phosphorus content of either the dried down isooctane extract or the "spots" scraped off the thin-layer chromatograms. One ml of the isooctane solution of the proteolipid from adrenodoxin was dried down in a warm round-bottomed digestion flask with a stream of nitrogen. The residue was digested with 2 ml perchloric acid until clear. The flask was cooled and 4 ml distilled water used to wash down the sides. It was placed in a boiling water-bath for 10 min and then cooled again. The digest was transferred to a 25 ml volumetric flask and made up to about 20 ml. 2 ml amidol-bisulphite reagent (1 g amidol and 20 g sodium metabisulphite in 100 ml water) and 1 ml 8.3% ammonium molybdate (Allen, 1940) were added and the volume made up to 25 ml. The contents were mixed and the absorption at 700 nm read after 10 min on a Unicam SP500 spectrophotometer. Blanks were processed right through the

digestion procedure and standard curves calibrated using 0-10 micromoles of potassium dihydrogen phosphate. Acid digests and subsequent manipulation for later determinations of the phosphorus content of the P450 proteolipid were as described by Chahl (1970) and Rouser, Siakotos and Fleischer (1966). Estimation of the adrenodoxin complex in isooctane was obtained / from its extinction at 325 nm following the approach used for the cytochrome c proteolipid (Das and Crane, 1964). The $E_{mM}^{325\ nm}$ used was 12.6 (Kimura, 1968).

Amino acid analysis of proteins

Proteins were hydrolysed in 6N-hydrochloric acid for 24 hours at 105°C and analysed by the usual automated ninhydrin techniques.

Anaerobic titrations

Anaerobic titrations were carried out in the apparatus devised by Foust et al. (1969) and constructed in this department by Mr. D. McDonald. Several cycles of evacuation and introduction of nitrogen were required to ensure anaerobiosis. During this, evaporation occurred from the solution in the spectrophotometer cell and was assessed by the change in extinction at the wavelength to be measured.

Miniature electrofocussing of proteins

The electrofocussing of purified protein was carried out by the method described by Koch and Bachx (1969). The principle of the method is that the protein of interest migrates due to an applied voltage in a continuous pH gradient formed of a series of peptides. When the protein reaches the pH equal to its isoelectric point it comes to rest as a narrow band. When the protein band is coloured the pH corresponding to its point of rest is easy to ascertain. With a colourless protein an enzymatic technique of location may be required since the peptide content of the medium would interfere with a protein determination. The apparatus was initially tested using haemoglobin and cytochrome c of known electrofocussing behaviour and found to give very fine resolution.

CHAPTER 3

RESULTS AND EXPERIMENTAL

SOME CHARACTERISTICS OF THE FLAVOPROTEIN COMPONENT

In the Introduction it was described how the steroid hydroxylating system of adrenal mitochondria was composed of at least three components, a flavoprotein, an iron-sulphur protein and a cytochrome P450 fraction. The subsequent discussion further revealed how much interest has been paid to the iron-sulphur protein, adrenodoxin, and the adrenal mitochondrial cytochrome P450 with the result that a considerable quantity of information has been gathered regarding these two segments of the electron-transport chain. However, surprisingly little work has been done on the flavoprotein fraction, It was decided therefore to attempt to develop a method of purification of the protein and study some of its characteristics and that of its cofactor, FAD, in this context.

Preparation of the flavoprotein

The method used is based on that described by Omura et al. (1965a, 1966, 1967) since it is economical to extract the content of iron-sulphur protein as well as the required flavoprotein from the mitochondrial enzyme preparation used. The mitochondrial protein (usually a lyophilised or acetone-dried powder, though occasionally freshly prepared mitochondria were used) was suspended in 2 volumes of Tris-HCl buffer pH 7.4, 0.1 M using an all-glass homogeniser. The protein was dialysed overnight against 50 volumes Tris-HCl 0.1 M and then sonicated in ice 5 x 1 min (Mark 5 on the MSE Ultrasonic Disintegrator) with cooling in ice for at least 1-2 min each in between bursts. It was then centrifuged at 100,000 g for 45 min. The clear supernatant was carefully decanted with minimal disturbance of the cloudy region immediately above the pellet. The supernatant was kept in the cold and the pellet was rehomogenised in Tris buffer using the all-glass homogeniser and sonicated as before. Centrifugation (100,000 g x 45 min) was carried out and the supernatants combined.

The pellet after re-extraction contained no detectable adrenodoxin but was rich in cytochrome P450 as was seen from its CO-reduced spectrum (the haem content of these pellets from an acetone powder source was measured as acid nonextractable iron using bathophenanthroline sulphonate and found to be 3.74 ng-atom haem Fe per mg protein). The combined supernatants were used as a source of adrenodoxin and adrenodoxin reductase.

The adrenodoxin and the flavoprotein were separated at this point by the use of DEAE-cellulose. The clear brown supernatant was applied to a column of DEAE-cellulose equilibrated in Tris-HCl, 0.1 M pH 7.4. With the passage of protein the adrenodoxin binds to the DEAE-cellulose and builds up into a brown band at the top of the bed. The further processing of the adrenodoxin component is described in Chapter 4. The flavoprotein sought, adrenodoxin reductase, (and NADH-DCPIP reductase as well) did not bind to the DEAE-cellulose under the above conditions (cf. Omura et al, 1966, found that the flavoprotein binds to DEAE-cellulose equilibrated in 10 mM phosphate buffer, pH 7.5 and is eluted by 40 mM buffer) and passed through the bed with the other unadsorbed proteins. A turbid milky fluid eluted first is followed by a haem-containing brown fraction containing a considerable amount of protein and the adrenodoxin reductase activity (Fig. 26).

In the early work on purification DEAE-Sephadex was tried and the yellow band which was described by Omura et al.(1966) (using DEAE-cellulose) was noticed. Fig. 17 shows a DEAE-Sephadex column with the reddish brown band of adrenodoxin and yellow flavoprotein band. However in work with DEAE-cellulose the bright yellow fractions were not found to coincide with the diaphorase activity and indeed the brightest yellow (and enzymatically inactive) eluates

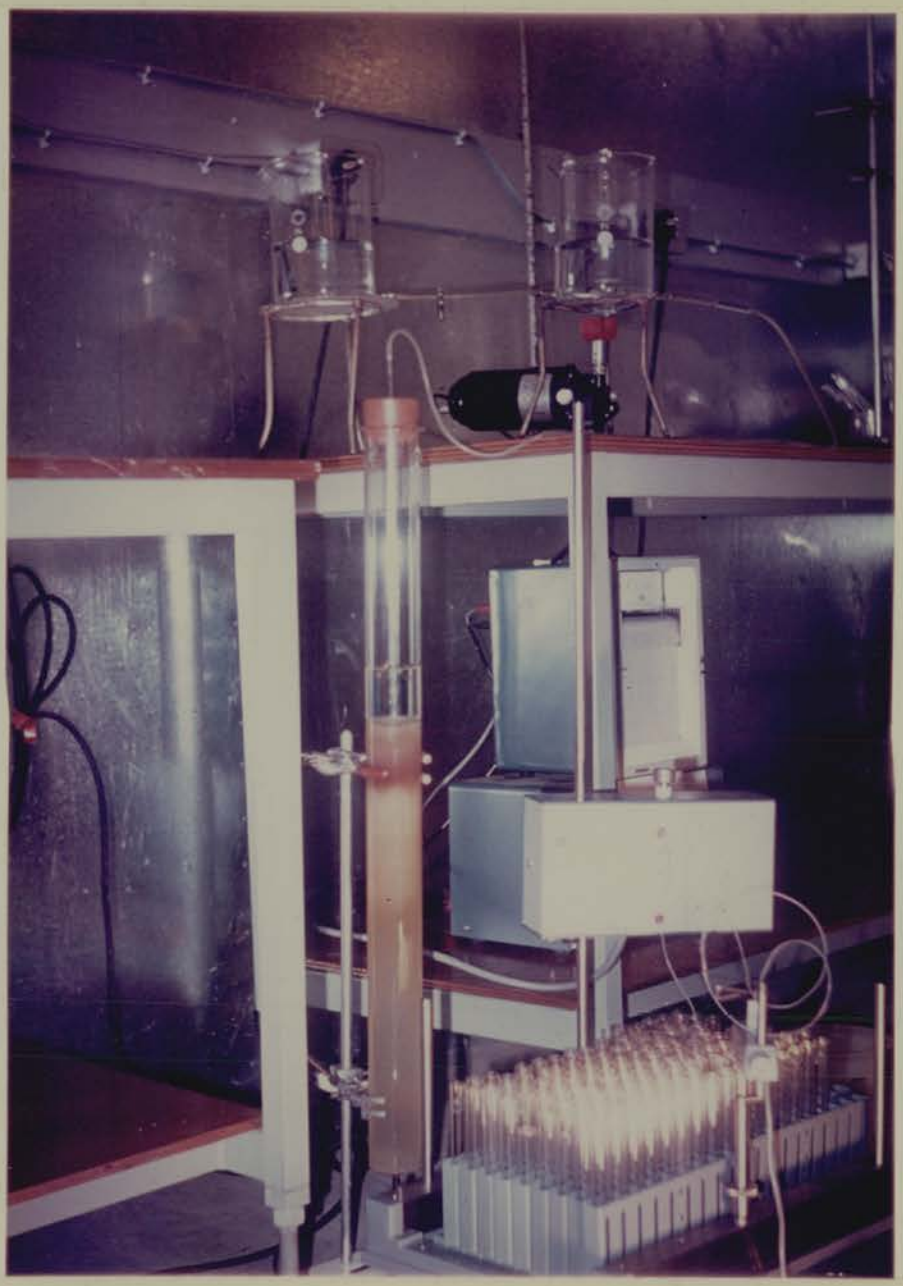


Fig. 17 DEAE-Sephadex chromatography of adrenal cortex mitochondrial enzyme extract

Adrenodoxin is adsorbed to the bed in a reddish-brown band (seen in column against upper arrow). Lower arrow shows position of the flavoprotein, adrenodoxin reductase.

were obtained between the flavoprotein and adrenodoxin fractions and after the elution of adrenodoxin at relatively high salt concentrations (Fig. 26). The effect of FAD on the diaphorase activity of colourless enzyme solutions is described later. The diminution of flow rate through DEAE-Sephadex during elution of adrenodoxin at high salt concentrations was so marked that after the initial trials with the gel, DEAE-cellulose was reverted to.7

The protein contained in the initial "front" (containing NADPH-DCPIP reductase but not NADPH-cytochrome c reductase) was pooled and subjected to fractionation using saturated ammonium sulphate solution buffered at pH 7.4 with Tris. The fraction precipitating between 35 and 60% saturation contained the flavoprotein as described by Omura et al.(1966). However it was found that haemprotein was not completely removed at this stage (cf. Omura et al., 1966).

The protein precipitated between 35 and 60% ammonium sulphate saturations was taken up in minimal Tris-maleate, 0.1 M, pH 6.5 and dialysed against the same buffer overnight. It was then passed through a column of CM-cellulose equilibrated at pH 6.5 in Tris-maleate 0.1 M. Under these conditions of protein and ion-exchanger the flavoprotein was not adsorbed on CM-cellulose and passed through unchanged although contaminants with different physical properties adhered to the exchanger.

The protein was passed through a column of Sephadex G100 in Tris-HCl 0.1 M, pH 7.4 and was recovered as a single symmetrical protein peak. The NADPH-DCPIP reductase activity paralleled the protein peak. (Sephadex G50 gel filtration performed at an earlier stage also showed the presence of a yellow fraction containing NADH-DCPIP reductase activity of a slightly lower molecular weight than the NADPH-DCPIP reductase. This did not correspond to the NADH-cytochrome c reductase observed by Omura et al.(1966).)

Gel-filtration on a calibrated Sephadex G75 column confirmed that the molecular weight is about 60,000 (Estabrook, 1966).

In view of the low specifity of the DCPIP-reduction assay technique the calculation of recovery figures of enzyme activity during purification was not undertaken. All operations were carried out at 0-4°C. The highest specific activity obtained was 923 nmol DCPIP reduced/min/mg protein in the presence of FAD.

~~Enzymatic assay of the flavoprotein~~

~~The assay of the flavoprotein was carried out by measuring the~~ reduction of dichlorophenolindophenol (DCPIP) spectrophotometrically at 590 nm. The reaction cuvette contained 0.1 ml DCPIP (0.3 mg/ml), 0.5 ml glucose-6-phosphate (25 mg/ml), 0.05 ml $NADP^+$ (7.5 mg/ml), 0.05ml glucose-6-phosphate dehydrogenase (10 u/ml), FAD (final concentration - 1 µM), active enzyme and Tris-HCl 0.1 M, pH 7.4 to 3 ml. All reagents are dissolved in buffer and the reaction started by the addition of flavoprotein to the sample cuvette. The slow nonenzymatic reduction of DCPIP by NADPH and reoxidation by air are counterbalanced by having these reagents in the reference cuvette and replacing the flavoprotein component with buffer. The assay is adapted from that described by Omura et al. (1966) significantly in the lower DCPIP-input and the inclusion of FAD. These adaptations significantly enhanced the reaction velocity. A DCPIP millimolar extinction coefficient of 19 was used (Savage, 1957).

The measurement of DCPIP reduction as an assay for adrenodoxin reductase is inadequate in several respects. It is non-specific since DCPIP is a known substrate for other flavoproteins (see Slater, 1966), inorganic salt concentrations can affect hydrogen ion transfer to DCPIP (Webb, 1963 p.835), FAD and DCPIP interact with each other directly (Yagi, Ozawa and Okada, 1959) as well as

with the enzyme and DCPIP interacts with NADPH generator especially in concentrated solution. Omura et al. (1966) have already commented on the harmful effect of preincubating the flavoprotein with DCPIP. The method is still the most convenient for monitoring the progress of a purification but checks need to be carried out periodically to ensure that the flavoprotein being isolated is adrenodoxin reductase. The checks which are available are the adrenodoxin-dependent NADPH-cytochrome c reduction activity (Omura et al., 1966), the reduction of the adrenodoxin chromophore in substrate quantities (Kimura and Suzuki, 1967), the reduction of cytochrome P450 in substrate quantities (Omura et al., 1966) and the reconstitution of the steroid hydroxylating system (Omura et al., 1966). The first is the most convenient, being rapid and economical of purified adrenodoxin and cytochrome P450 enzyme preparations and was routinely used. The requirement of both the flavoprotein and adrenodoxin preparations ~~for NADPH-cytochrome c reduction was verified initially.~~

Protein content of flavoprotein preparations

The biuret (Gornall, Bardawill and David, 1949) and Lowry (Lowry et al., 1951) methods are widely used for protein determination. However the method developed by Warburg and Christian (1941) of estimating the protein content from the comparison of the spectral extinction at 260 nm and 280 nm of the enzyme solution is often a method of choice being rapid and especially since it does not consume purified enzyme. However purified proteins have different contents of the aromatic amino acid residues on which this method depends. It was therefore advisable to confirm experimentally whether results obtained by this method for adrenodoxin reductase were comparable to those obtained by a "standard" method.

Adrenodoxin reductase sample (no.of determinations-two)	Protein content by $E^{260nm}:E^{280nm}$ method	Protein content by Lowry method
50 µl	55 µg	52 µg

The determination was made and the average of the values obtained are seen to correspond reasonably well having used a Lowry method calibrated with bovine serum albumin and an $E^{260nm}:E^{280nm}$ nomogram based on extinction coefficients for enolase and nucleic acid.

Nitrogen content of purified flavoprotein

This measurement was performed on digests of a specimen of flavoprotein and showed adrenodoxin reductase to contain 116 µg N per mg protein.

Disc-gel electrophoresis of purified flavoprotein

The flavoprotein prepared by the method described gave single symmetrical protein peaks on gel-filtration and ion-exchange chromatography. Its purity was further examined by disc-gel electrophoresis (see Methods). Electrophoresis at pH 7.4 and at pH 6.0 (80 volts for 5 hours) were unsuccessful, little or no migration into the gel occurring but electrophoresis at a higher pH was successful. The flavoprotein (previously incubated with FAD and dialysed) in Tris-HCl pH 9.0, 20 mM travelled 2 cm towards the anode in 2 hours under a current of 300 volts across a 10% polyacrylamide gel 5.2 cm long. At the end of electrophoresis a sharply defined bright yellow band was observed which coincided with the protein band resulting from staining (Fig. 18).

Attempts to locate the DCPIP-reducing activity after electrophoresis (cf. O'Barr,1969; O'Barr and Smith, 1969; Skyring, Miller and Purkayastha, 1970) were not successful.

Fluorescence of the flavoprotein

Table IV shows that the fluorescence properties of pure adrenodoxin

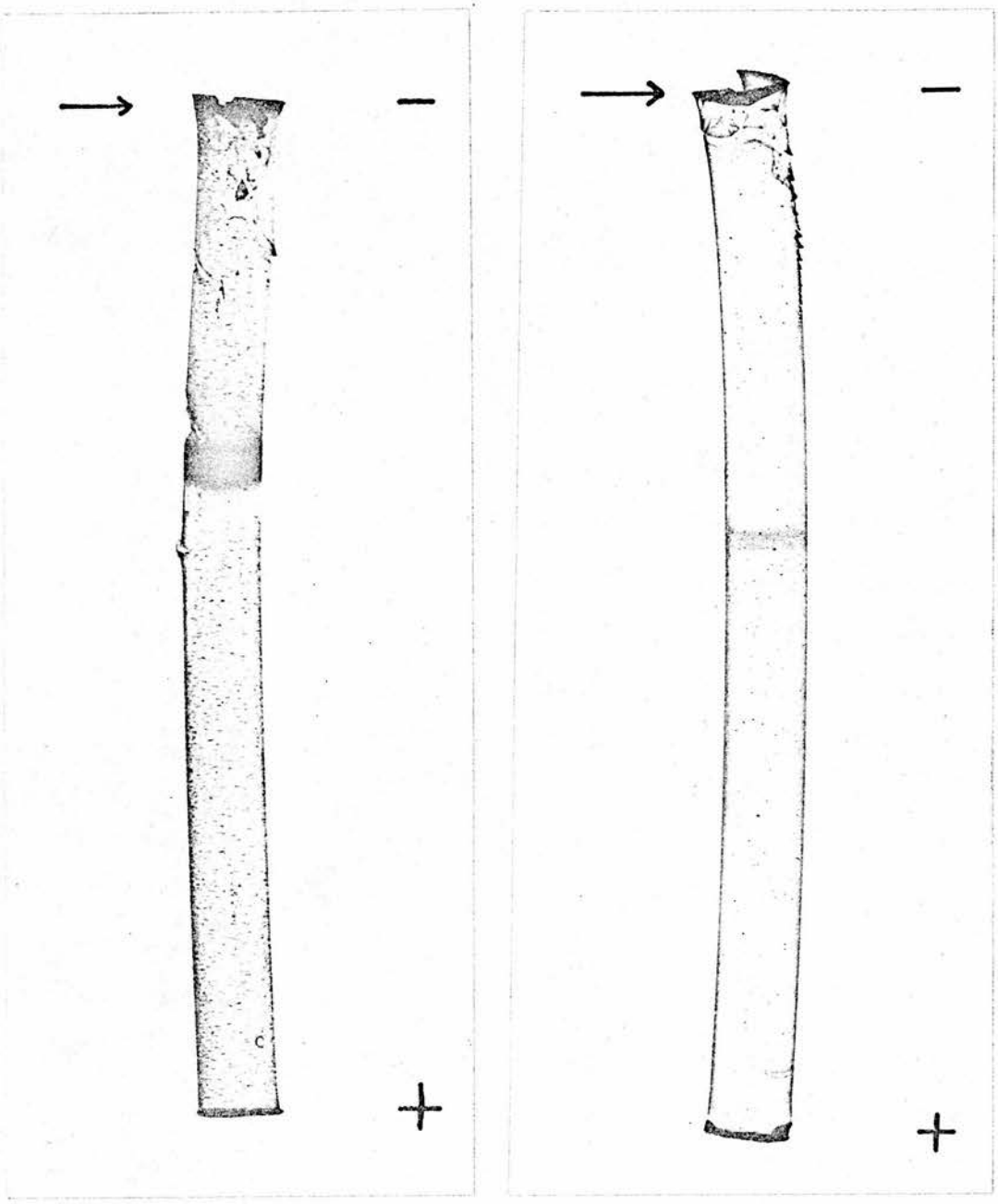


Fig. 18 Disc-gel electrophoresis of adrenodoxin reductase

Origin indicated by arrows; 10% polyacrylamide gel, 5.2 cm long; other experimental details as described in the text.

Table IV **Fluorescence characteristics of adrenodoxin reductase**

Excitation wavelength (nm)	Emission wavelength (nm)
290	334
395	523, 791
465	520

reductase are similar to those of other flavoproteins.

pH dependency of flavoprotein activity

Fig. 19 shows the pH dependency of the DCPIP-reducing activity of the flavoprotein. DCPIP can be nonenzymatically reduced by NADH in acidic solutions (Rafter and Colowick, 1955). However at the pH for optimal activity of this flavoprotein of about 6.5 the assay is still reliable and Rafter and Colowick (1955) have in fact used the method to determine the pH profile of 3-phosphoglyceraldehyde dehydrogenase with an optimum pH of 5.0. Equimolar Tris-maleate (NaOH) buffers were used and the exact pH values were determined with a glass-electrode.

Miniature electrofocussing of the flavoprotein

The protein band after miniature electrofocussing (see Methods) was not visible so the aliquots of solution resulting from division of the frozen column were assayed for DCPIP-reducing activity under buffered conditions. The pattern of the activity location compared to the pH gradient (Fig. 20) indicates that whereas a minor component of DCPIP-reducing activity migrates to pH 4.6-5.5, the principal component is probably near pH 8.9.

Investigations using the cofactor FAD

The coenzyme of the flavoprotein is FAD (Estabrook, 1966). Studies were made of the effect of FAD in relation to enzyme activity.

(a) Activation of adrenodoxin reductase with FAD

A colourless enzyme preparation resulting from the usual ammonium sulphate fractionation and ion-exchange chromatography preparative procedure was found to contain practically no NADPH-DCPIP reductase activity. However preincubation of the enzyme with FAD (23 nmol FAD per mg protein) resulted in the enzyme preparation showing considerable activity. Fig. 21 shows the activity with protein only,

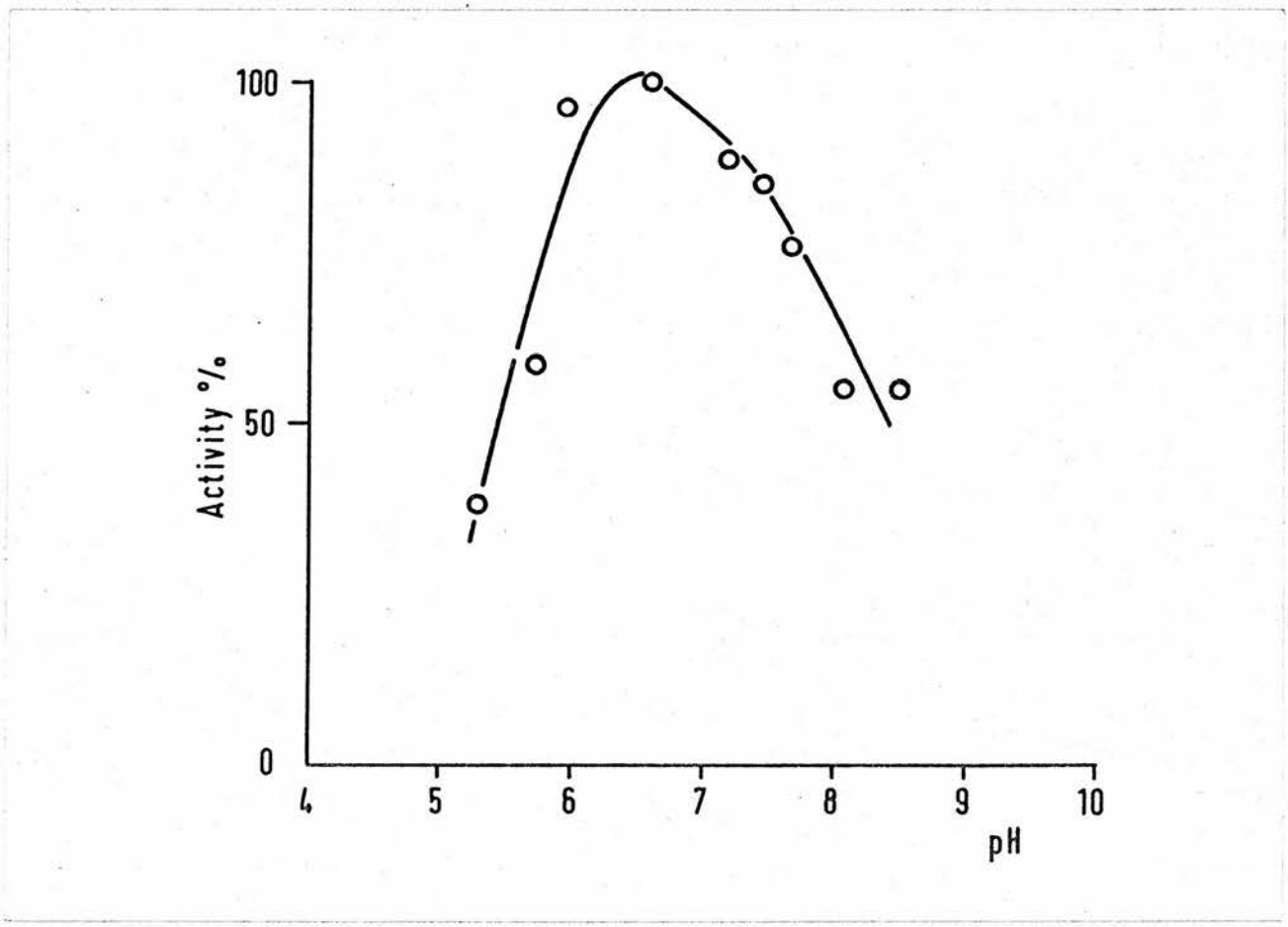


Fig. 19 The relative rates of reduction of DCPIP by adrenodoxin reductase at different pH values

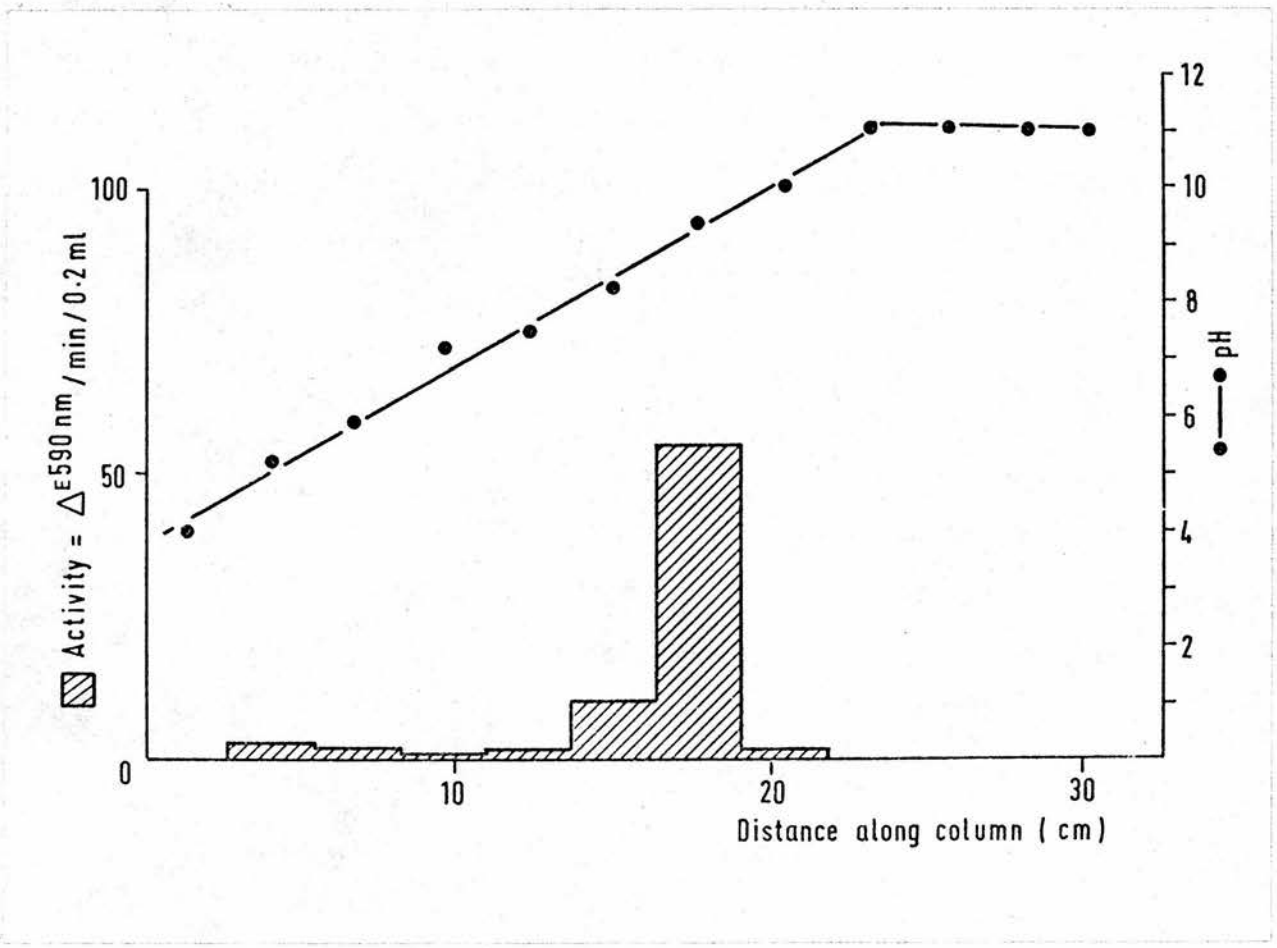


Fig. 20 The location of adrenodoxin reductase following miniature electrofocussing

or neither protein nor FAD to be negligible by comparison with activity noted with enzyme and FAD present. Extensive preincubation was not found to be necessary and FAD was not effective in the absence of enzyme. It was found that FAD added to a reaction mixture to which enzyme and substrate had already been added resulted in restoration of comparable activity (Fig. 21).

It was found that FMN also gave a marked stimulation of enzyme activity. It was shown that this effect may have been due to the presence of FAD as a contaminant.
~~and FMN as in the NADH-dehydrogenase concerned in camphor lactonisation~~ (Trudgill, Du Bus and Gunsalus, 1966a). However the FMN (Sigma) used carried no claim to be free of FAD so it was decided to examine this by paper chromatography. The system used was descending paper chromatography with n-butanol:acetic acid:water:: 50:12:50 upper phase as used previously for separating imidazole derivatives (Wickramasinghe, 1970) which was slightly different from the ascending paper chromatography in n-butanol:acetic acid: water::4:1:5 upper phase described by Huennekens and Felton (1957) for separating FAD and FMN. These compounds are located on the developed chromatogram by their fluorescence in ultraviolet light.

The chromatography showed the FMN to contain about six fluorescing components of which that migrating with FAD is in enough proportion to activate the flavoprotein. The average Rf values for FAD and FMN shown below in the system used appear to show an improved separation over the published figures.

	Rf	
	FAD	FMN
Huennekens and Felton (1957)	0.05	0.13
~~Present system(average values)~~	~~0.038~~	~~0.162~~

(b) **Effects of a range of FAD concentrations on the DCPIP-reducing activity of the flavoprotein**

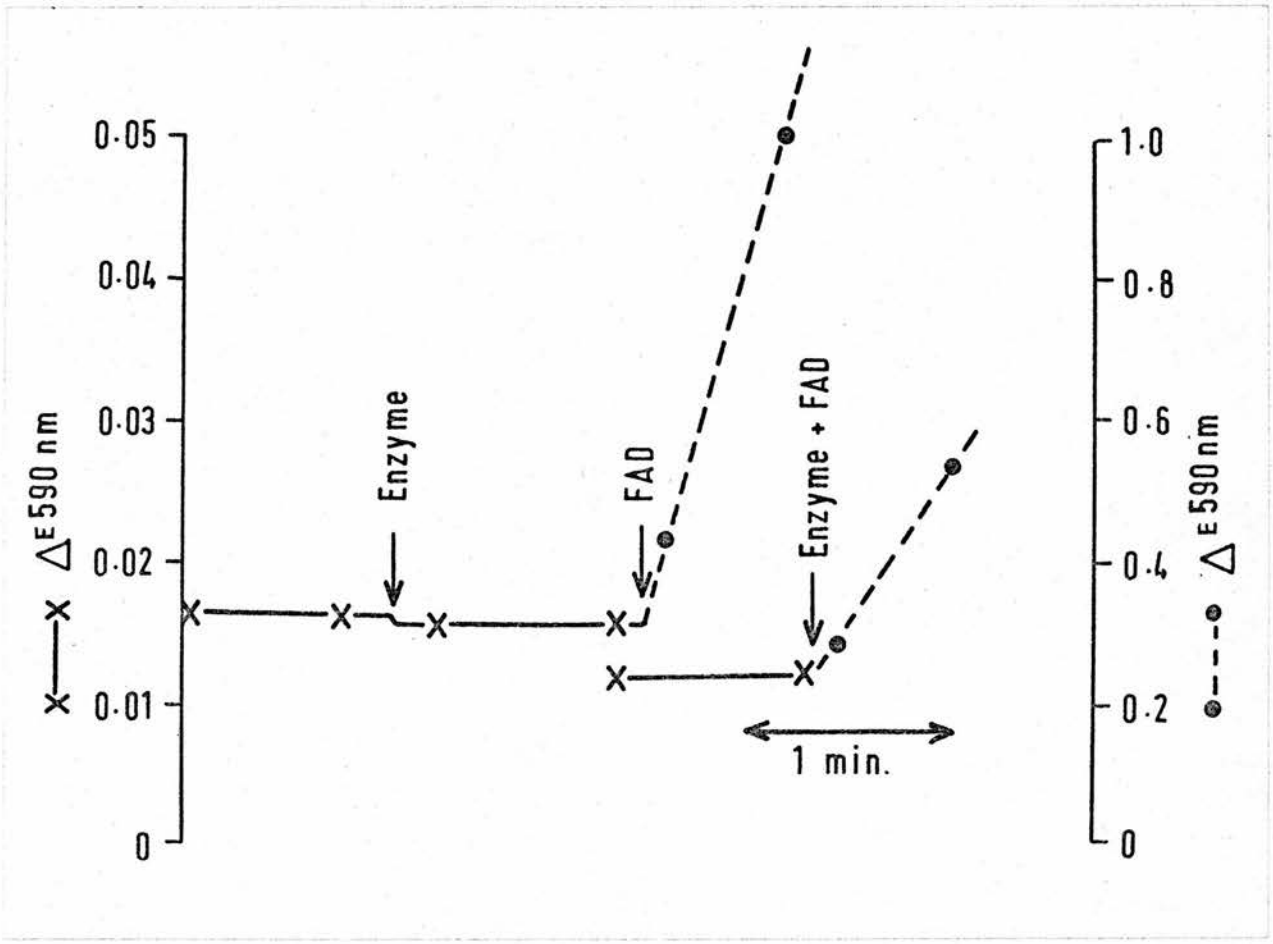


Fig. 21 The activation by FAD of the DCPIP-reducing activity of adrenodoxin reductase

The solid lines show measurements made on the light extinction scale shown on the left while the activity of the enzyme following addition of FAD (dashed lines) was measured on the scale indicated on the right. The method of assay was as described in "Methods" and 23 nmol FAD per mg protein were added at the indicated points.

A systematic examination of the effect of FAD over a range of concentrations was made both to find an optimal amount to be added to the routine DCPIP-reduction assay and to see the possible effects of addition of excess. Fig. 22 shows that enzyme activation takes place at very dilute concentrations of FAD with an optimal concentration / at around 1-10 μM FAD and inhibition being observed at higher concentrations. FAD (1 μM) is therefore now added to the routine DCPIP assays irrespective of protein concentration in the range of 60 μg to 1 mg per ml.

The inhibition by excess FAD could be due to excess flavin blocking the binding-sites for DCPIP by binding onto the enzyme. A detailed examination was therefore made of the effects on enzymic activity of varying the concentration / of DCPIP in the presence of different concentrations of FAD. A competitive inhibition could not be demonstrated possibly due to the nonenzymatic interaction of FAD with phenolic substances (Yagi, Ozawa and Okada, 1959) in addition to their competing for the coenzyme site and with the substrate. However from this study it was found that the use of ~~0.1 ml~~ DCPIP (0.1 μmol) per 3 ml assay gave a higher reaction velocity than that of ~~0.2 ml~~ 2 μmol DCPIP stated by Omura et al.(1966). This former concentration / of DCPIP was therefore adopted for routine assays.

The FAD chromophore at 450 nm can be bleached with dithionite. Studies utilising the reduction of the FAD chromophore have been reported by Hosokawa and Stanier (1966), Katagiri et al.(1965) and Maki et al.(1966). An attempt was made to see if the excess FAD acts as an electron-accepting substrate by utilising the flavoprotein assay, omitting the DCPIP, and measuring changes in extinction at 450 nm. No enzymatic reduction of the FAD chromophore was detectable even over several hours.

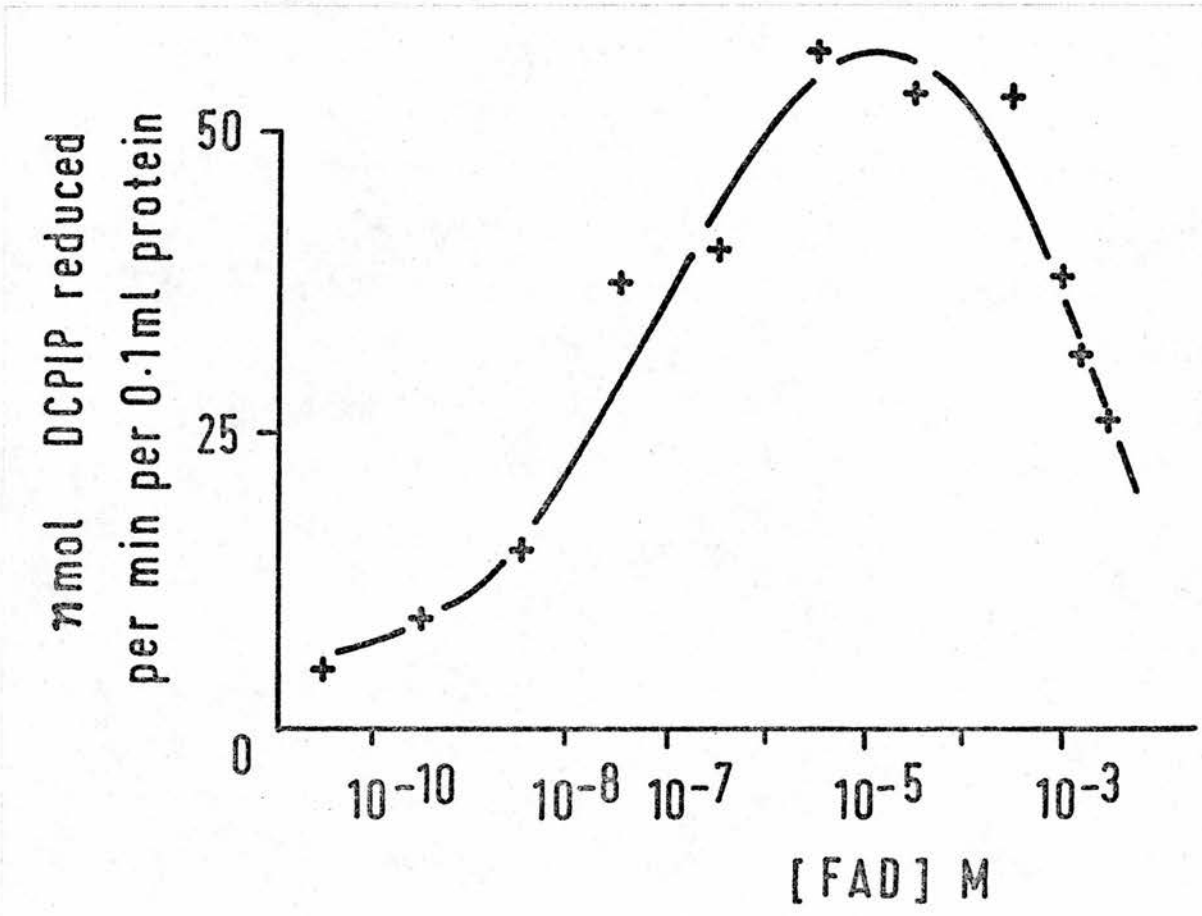


Fig. 22 Variation of rate of reduction of DCPIP by adrenodoxin reductase with concentration of FAD

The rate is shown expressed per 0.1 ml of a highly purified enzyme preparation (1.9 mg protein per ml)

(c) The anaerobic NADPH-FAD reducing activity of adrenodoxin reductase

The possibility that NADPH-FAD reduction may however be demonstrable under strictly anaerobic conditions was examined using the apparatus devised and described by Foust et al. (1969). Solutions of NADPH (~~not generator~~) and FAD were estimated according to published extinction coefficients for the oxidised and reduced forms (Beinert, 1960; Penzer and Radda, 1967; Whitby, 1953). A known amount of enzyme-containing FAD solution was introduced into the cuvette, the deaeration cycles carried out, the initial reading at 450 nm taken and the previously deoxygenated NADPH solution titrated in. The decrease in extinction at 450 nm (after correction for dilution) shows that bleaching of FAD does take place under these conditions (Fig. 23). Assuming that in the initial stages of the titration the FAD is converted to its fully reduced form, the initial stoichiometry from the change of extinction at 450 nm shows that the addition of 2.34×10^{-1} µ mol of NADPH results in the reduction of 1.98×10^{-1} µ mol of FAD. This is a reasonable stoichiometry since it is likely that some of the semi-reduced form would also result. Other experiments confirmed that the reaction depended on the presence of the enzyme. Fig. 23 shows also that a momentary exposure to air causes partial reoxidation of the FAD chromophore which accounts for the earlier inability to demonstrate reduction of FAD under aerobic conditions.

(d) The effect of FAD on cholesterol side-chain cleavage and 11β-hydroxylation

The work described above shows that the DCPIP-reducing activity of purified flavoprotein is inhibited by excess FAD. It was decided to examine the effect of low and high concentrations of FAD on the steroid hydroxylating activity of adrenal mitochondrial protein.

De Luca, Weber and Kaplan (1956) found that a variety of (rat)

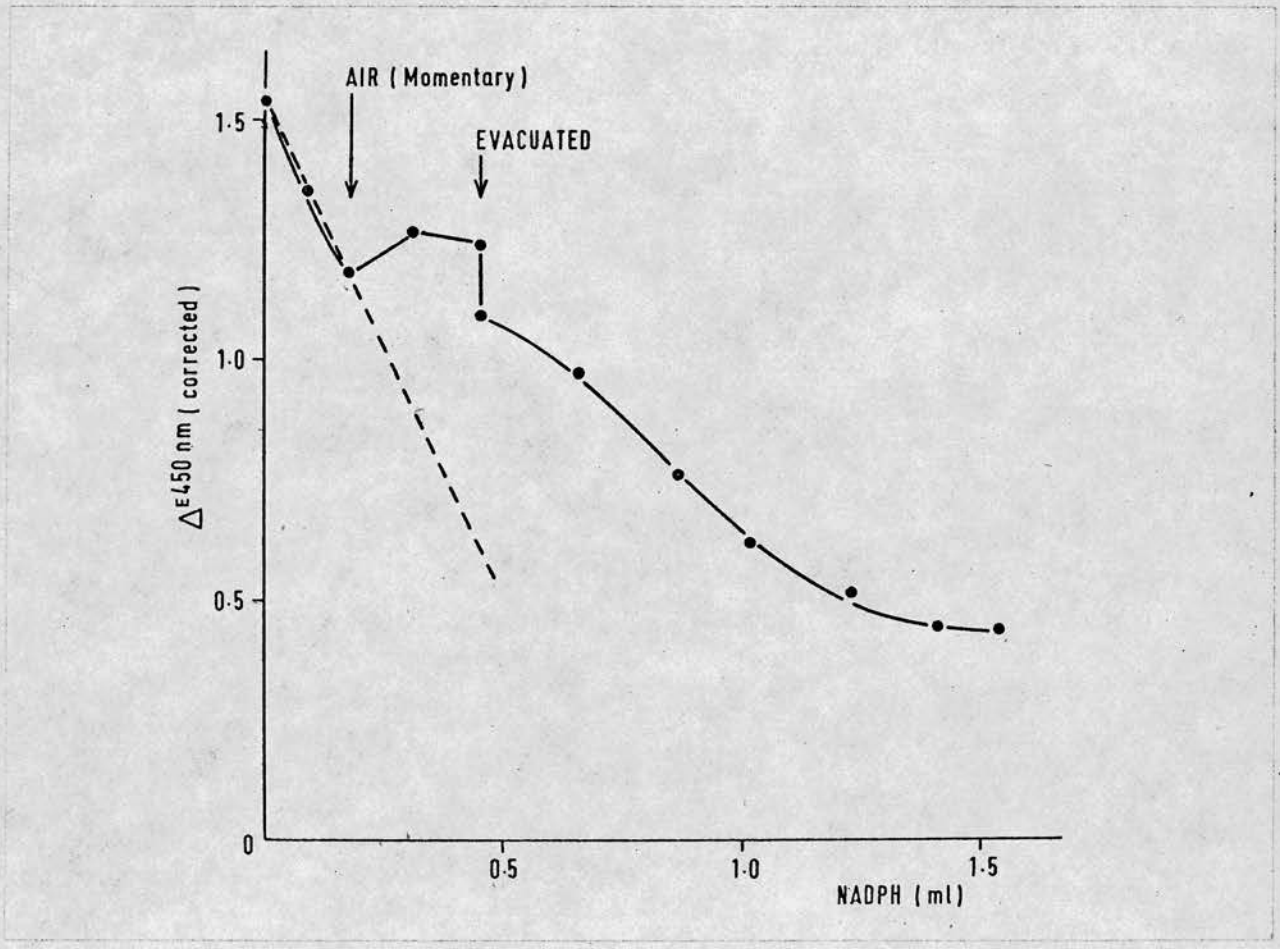


Fig. 23 The reduction of FAD by NADPH and adrenodoxin reductase under anaerobic conditions

The reaction mixture contained FAD (0.27 µmol), enzyme (1.1 mg) and Tris-HCl (0.275 mmol, pH 7.4) in 2 ml. The titrant was 0.47 mM-NADPH in 0.1 M-Tris-HCl, pH 7.4.

organs contained about 8-55 µg FAD per g fresh tissue and King et al. (1962) found that heart protein contains 0.125-0.3 µ mol FAD/g protein. For the work described here the soluble (100,000 g x 30 min) supernatant from the homogenisation of a mitochondrial acetone powder was used and within a range of about 1.5×10^{-4} µ mol to 1.5 µ mol FAD added per mg of soluble protein (or about 4.8×10^{-5} to 4.8×10^{-1} µ mol FAD/ml incubation mixture). Fig. 24 shows that although preparation of an acetone powder may well be expected to result in loss of FAD, the addition of FAD does not show an appreciable enhancement of the rate of cholesterol side-chain cleavage. At high concentrations however a marked inhibition takes place. Similar effects are observed on examining the effect of FAD on the 11 β-hydroxylation of DOC by enzyme preparations from solubilised adrenal mitochondria (Fig. 25) at similar FAD to protein ratios.

Amino acid composition

The amino acid composition of the flavoprotein (of a purity as shown in Fig. 18) is given in Table V. The figures are expressed as percentages of each species of the total amino acid content pending a precise figure for the molecular weight of the protein as determined by several different methods.

Summary

1. A method of preparation of pure adrenodoxin reductase is described together with details of its examination and estimation.
2. The fluorescence of the protein is described.
3. The pH dependency of the enzymatic activity is given. The optimal activity was observed at about pH 6.5.
4. Miniature electrofocussing of the protein showed that its

isoelectric point is about pH 8.9 unlike the acidic adrenodoxin and cytochrome P450.

5. Investigations were made of the effect of the cofactor FAD on the flavoprotein and steroid hydroxylating system activity. It was found that FAD

   (a) in low concentrations activated the reduction of DCPIP by the flavoprotein and in high concentrations inhibited it,

   (b) was reduced by NADPH in the presence of flavoprotein and reoxidised by molecular oxygen, and

   (c) inhibited steroid hydroxylation in high concentration.

6. The percentage amino acid composition of adrenodoxin reductase is given.

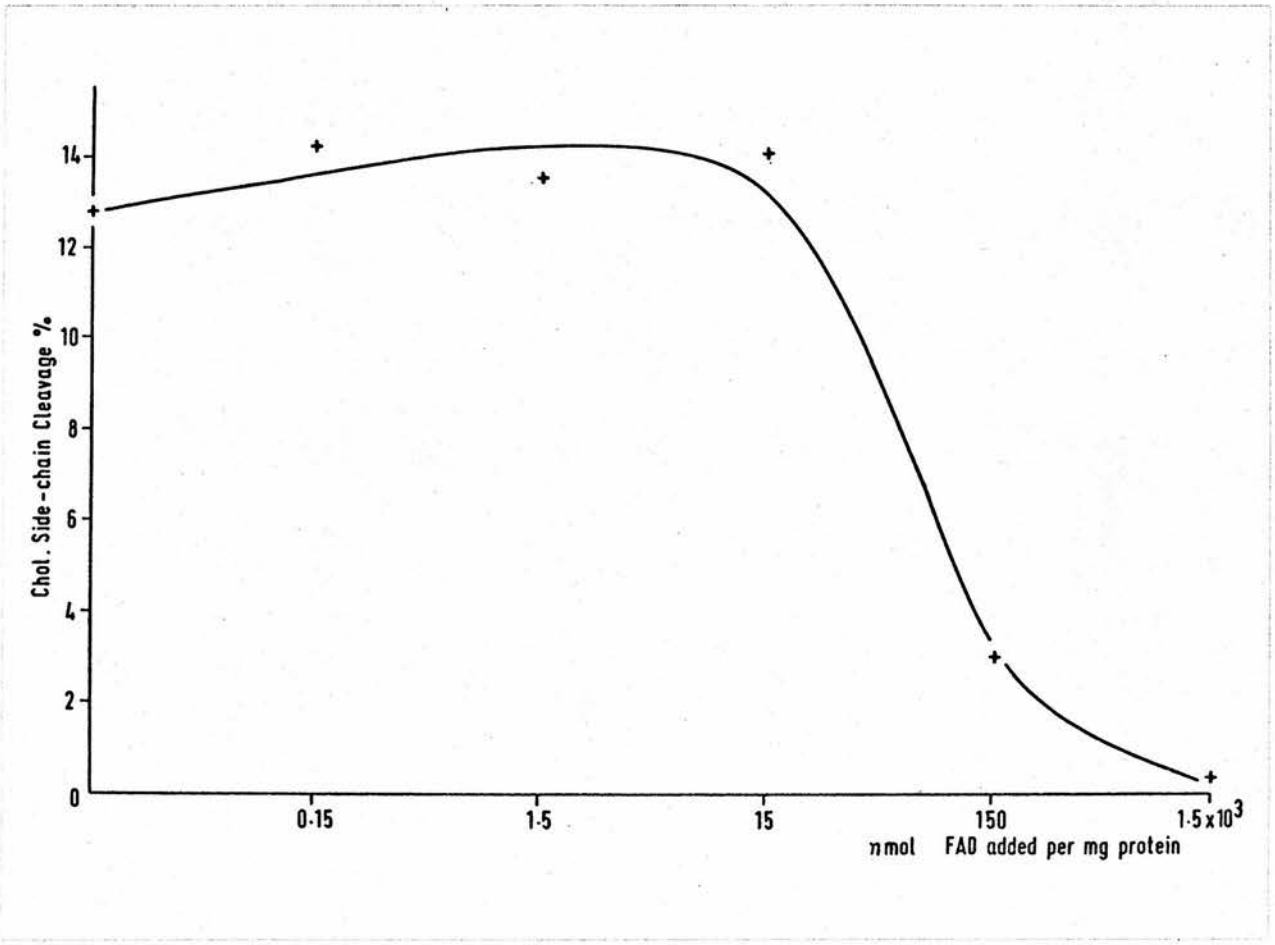


Fig. 24 Effect of exogenous FAD on cholesterol side-chain cleavage activity assays in vitro

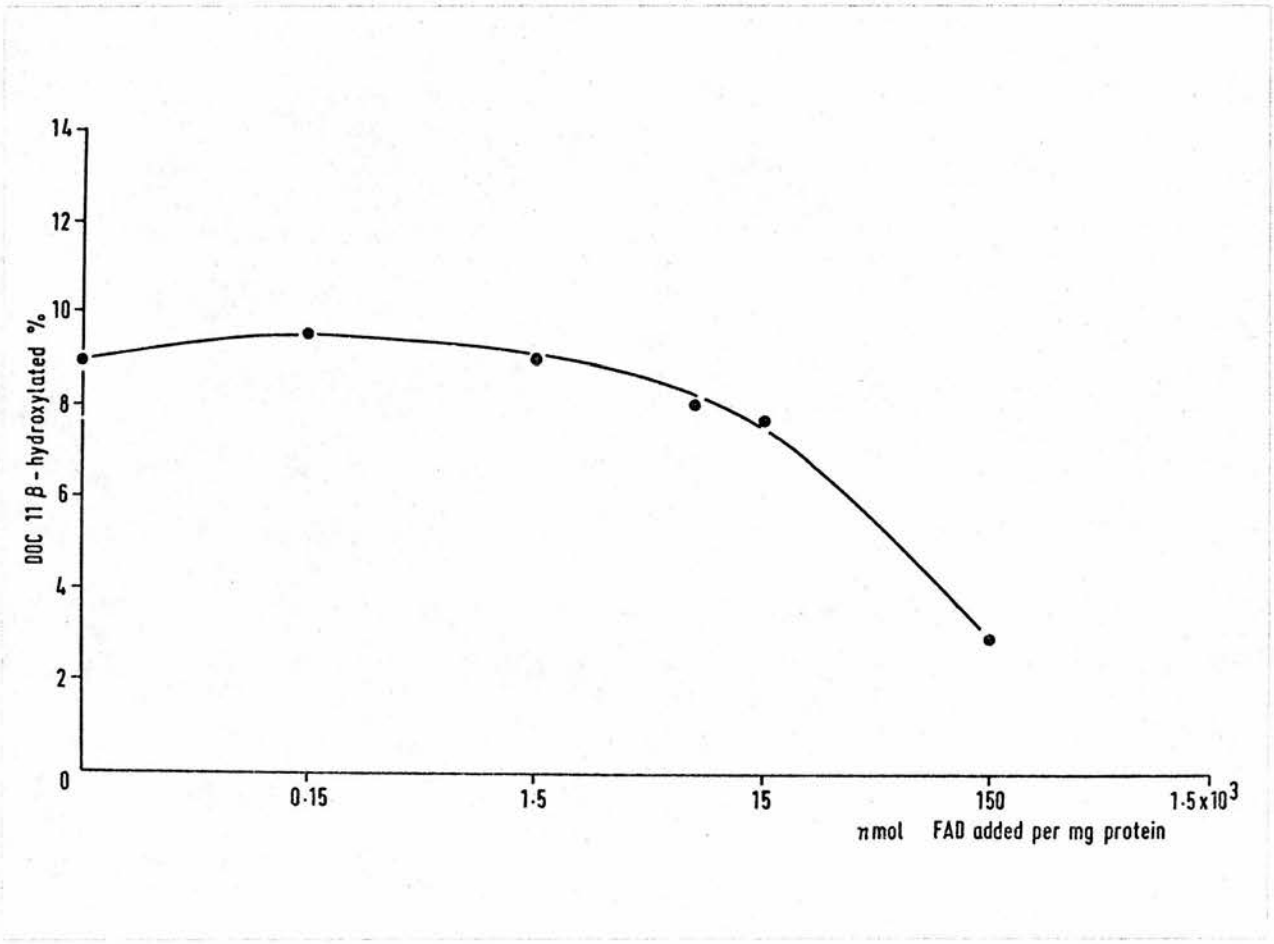


Fig. 25 Effect of exogenous FAD on DOC 11 β-hydroxylation activity assays in vitro

Table V Amino acid composition of adrenodoxin reductase

Residue	Percentage content
Asx	8.66
Thr	5.61
Ser	5.86
Glx	8.77
Pro	6.82
Gly	9.39
Ala	8.64
Cys($\frac{1}{2}$)	n.d.(2.0?)
Val	8.20
Met	1.99
Ile	4.85
Leu	9.11
Tyr	2.24
Phe	3.91
His	2.41
Lys	6.95
Trp	n.d.
Arg	4.61

(n.d. = not specifically determined)

CHAPTER 4
RESULTS AND EXPERIMENTAL

INVESTIGATIONS ON ADRENODOXIN

Purification

Several methods of purification of adrenodoxin have been described, many comparable with methods for other ISP^{s} (see Introduction). A novel method described by Kimura (1968) depends on the batch-wise adsorption of the acidic adrenodoxin from a clarified homogenate of whole adrenal glands onto DEAE-cellulose which is then further processed. Purifications of adrenodoxin by this method (Kimura and Huang, 1970; Kimura, Tasaki and Watari, 1970; Orme-Johnson and Beinert, 1969c) gave the relatively high yields of about 90 mg protein per kg adrenal glands compared to some early yields of about 10 mg protein per kg adrenal glands (Kimura, 1968; Omura et al, 1967). The newer method was tried but not found to be very satisfactory owing to difficulties in recovering the DEAE-cellulose and separating adrenodoxin from other brown pigments. Consequently this method was not used routinely since it also did not allow for the isolation of the flavoprotein, adrenodoxin reductase.

The preliminary steps of the purification were similar to those employed for the flavoprotein and have been described(Chapter 3). The remaining steps of the large scale purification procedure of adrenodoxin developed in the course of this study are now described. The protein containing adrenodoxin was adsorbed as a dark-brown band on a DEAE-cellulose column which was subjected to a linear elution gradient of 0.2 to 0.8 M-KCl in 0.1 M-Tris-HCl pH 7.4 buffer. The volume of the complete gradient was about four times the total volume of the ion-exchanger bed. The brown band started to be eluted and to move downwards from a KCl concentration of about 0.25-0.3 M but total desorption required about 0.5 M total salt concentration. The eluted protein

solution was collected over usually about 100-120 fractions using an LKB 7000A "Ultra Rac" fraction collector. The progress of desorption of protein was followed by recording the eluate's extinction at 280 nm using an LKB 8300A "Uvicord II" absorptiometer and a 6520H recorder. At this stage in the purification the presence of other proteinaceous contaminants which were desorbed close to adrenodoxin and the low content of aromatic amino acids in the latter protein combined to produce the unusual phenomenon of the 280 nm extinction trace showing a dip where the brown protein is found. Fig. 26 shows a typical elution pattern where the flavoprotein fraction had not been washed out beforehand but an eluting gradient applied. The elution of adrenodoxin did not result in increased extinction at 280 nm.

The salt concentration of the pooled adrenodoxin fractions was lowered by a short dialysis against or dilution with 50 mM-Tris-HCl and the protein concentrated by adsorption on to a very small quantity of equilibrated DEAE-cellulose which was centrifuged down and desorbed with KCl in Tris buffer. The desorption was repeated to recover any protein occluded in the cellulose. The concentrated protein solution was applied to a Sephadex G75 column and chromatographed in 0.1 M-Tris-HCl. The alternate ion-exchange chromatography and molecular sieving by gel-filtration was repeated until a single symmetrical protein peak coincided with the peak of extinction at 415 nm and the $E^{415nm}:E^{280nm}$ ratio peak as described in the Introduction. ~~Kimura's methods of preparation has regularly yielded protein of $E^{415nm}:E^{280nm}$ of about~~ 0.76 and Omura et al.(1967) obtain about 0.8, ~~while~~ Orme-Johnson and Beinert (1969c) have reported preparations with $E^{415nm}:E^{280nm}$ ratio as high as 0.88. ~~These~~ results conflict with molar extinction coefficients ~~at 414 nm and 276 nm published by Kimura (1968) which give a maximal $E^{414nm}:E^{276nm}$ ratio of 0.754.~~ Preparations obtained in the course of

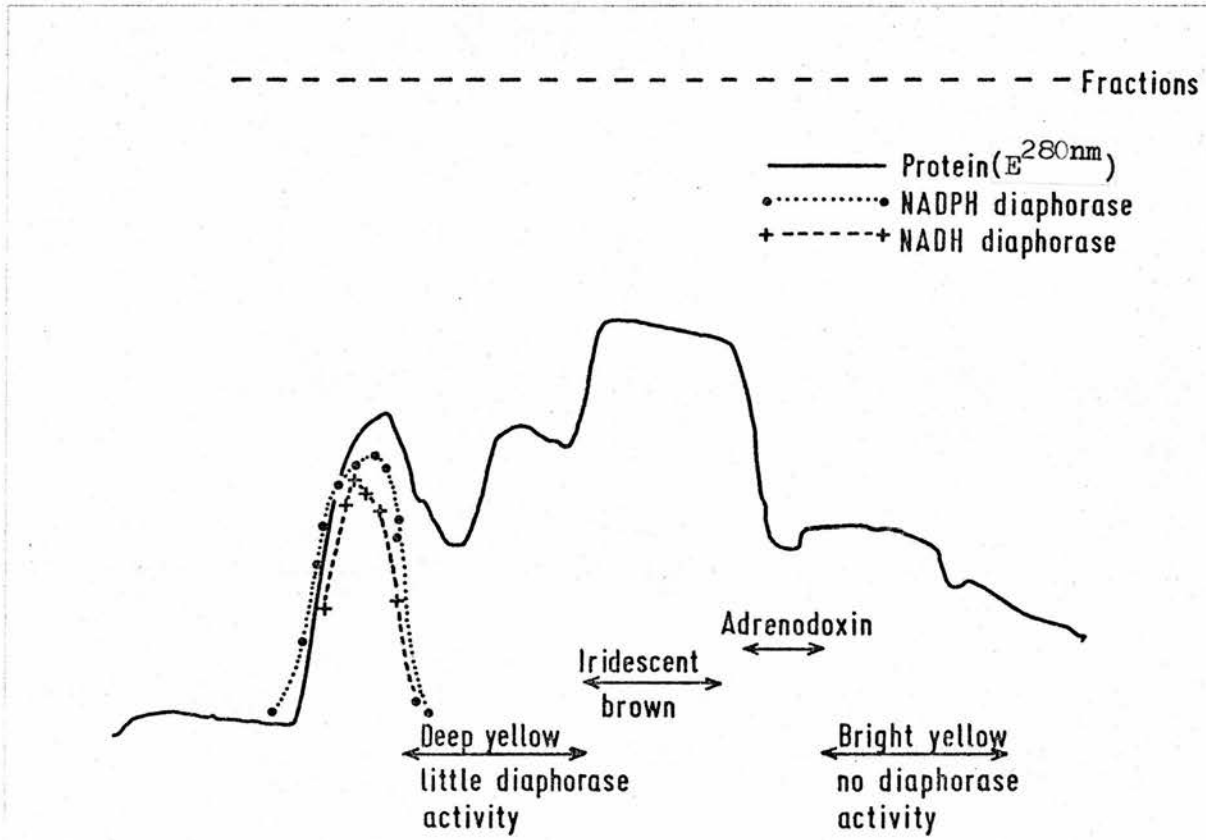


Fig. 26 DEAE-cellulose chromatography of an adrenal cortex mitochondrial enzyme extract

A linear elution gradient of 0.2 to 0.8 M-KCl in 0.1 M-Tris-HCl buffer, pH 7.4 was used for desorption. The desorption of protein was monitored by the change in extinction at 280 nm. The diaphorase activities were located by measurement of rate of DCPIP-reduction.

the present work gave $E^{415nm}:E^{280nm}$ ratios of 0.81 and yields of about 50 mg protein per freeze-dried mitochondria equivalent to one kg adrenal glands are obtained.

Ammonium sulphate precipitation has been tried both for fractionation and concentration but even under the most carefully controlled pH conditions and using Enzyme-grade Ammonium sulphate, denaturation of the protein has been experienced. Similarly the protein at later stages of the purification tended to show instability to lengthy dialysis.

Mortenson(1964b) has made use of the solubility of ferredoxin in 50% acetone in a preparative procedure. Experience with stepwise fractionation with redistilled acetone to 60% showed that adrenodoxin tended to precipitate in the 0-15% and 15-30% acetone fractions with the solutions kept cooled with solid carbon dioxide. However the recoveries were low and the procedure could not be used as a preparative step. Attempted purification with Protamine 1% was also unsuccessful.

Independent work developed in the course of this investigation over the last three years has thus resulted in a preparative method for adrenodoxin similar to Procedure A of Kimura (1968). The method has given consistently reliable results with good yields of a pure protein. Experience indicated the instability of adrenodoxin on precipitation in ammonium sulphate solution. However repeated ion-exchange and gel-filtration chromatography proved to be a safe and relatively rapid means of producing a good yield of highly purified adrenodoxin. All operations of the described method of purification were carried out at 0-4°C.

Determination of the progress of purification and protein determination

The methods available for determining the progress of purification

of iron-sulphur proteins have been discussed (see Introduction). The ratio $E^{415nm}:E^{280nm}$ is also used as a criterion of purification of adrenodoxin and as has been discussed above the best preparations obtained usually have a ratio of 0.8 to 0.81. Omura et al.(1966) reported the absolute requirement of both adrenodoxin and adrenodoxin reductase for the enzymatic reduction of cytochrome c by NADPH. This was confirmed in the course of the present work and is a useful rapid means of checking the enzymatic functioning of preparations with a high $E^{415nm}:E^{280nm}$ ratio.

In the work on adrenodoxin reductase (Chapter 3)it was found that the measurement of the purified protein by the Lowry method or that based on its extinction at 260 nm and 280 nm gave similar results as to protein content. However the low aromatic amino acid content of adrenodoxin made the results obtained by the two methods widely different in this case. The biuret and Lowry methods were however comparable and the biuret method was routinely used.

	by $E^{260nm}:E^{280nm}$ method	by biuret method	by Lowry method
Protein concentration (mg protein/ml) (no.of determinations-two)	2.22	4.84	4.8

The concentration of purified adrenodoxin was estimated from the molar extinction coefficients published by Kimura (1968); of which $E^{414nm} = 9,800\ cm^{-1}M^{-1}$.

The nitrogen content of adrenodoxin ($E^{415nm}:E^{280nm} = 0.79$) was determined to be 149 μg N/mg protein after digestion in sulphuric acid, perchloric acid and hydrogen peroxide for about 13 min and using phenol and hypochlorite reagents for the determination.

Possibility of the existence of isoenzymes of adrenodoxin

The separation of the cytochrome P450 components of cholesterol side-chain cleavage and 11 β-hydroxylation has been reported recently

by Jefcoate, Hume and Boyd (1970). The present work (Chapter 6) indicates two different Km values for NADPH for cholesterol side-chain cleavage and 11 β-hydroxylation. Assuming that adrenodoxin reductase, adrenodoxin and cytochrome P450 are the only types of enzymes involved in the different adrenal mitochondrial steroid hydroxylations, this could indicate the existence of two different flavoproteins. These aspects will be discussed elsewhere. It was decided also to investigate if more than one form of adrenodoxin could be identified.

Electrophoresis of adrenodoxin on cellulose acetate was first used but since definition was not sharp, polyacrylamide disc-gel electrophoresis was carried out. Samples of adrenodoxin (E^{415nm}: E^{280nm} = 0.76) were electrophoresed at pH 7.4 and pH 8.9 (5 mM Tris buffer). Staining the gels with the protein stain Naphthalene black 10B showed the presence of about three protein bands. However one or two of these bands may have been apoprotein forms resulting from partial (or total) loss of iron and labile suphur content. The "iron-stain" devised (see Methods) was applied therefore to a specimen which had been gel-electrophoresed in parallel. A diffuse pink colour developed in the region of the protein components, due to iron liberated from the protein but the definition was not sufficiently distinct to identify separate bands.

Owing to the difficulties in obtaining decisive proof by electrophoresis as to the existence of adrenodoxin isoenzymes, attempts were made to see whether the successive fractions of adrenodoxin eluted from a DEAE-cellulose column show a (gradual) transition in functional specificity in steroid hydroxylations. A problem encountered here is that adrenodoxin is the middle protein of a chain of at least three enzymes (viz. adrenodoxin

reductase, adrenodoxin and cytochrome P450). The proteins at either end of the chain may be specific (for cholesterol side-chain cleavage, 11β- and 18-hydroxylations) and, while the separation of the flavoprotein activities has not been accomplished, the purified and separated specific cytochrome P450 fractions are unstable. The other obstacle is that cholesterol side-chain cleavage and DOC 11β-hydroxylation are markedly affected by salt concentrations and extraneous proteins (see Chapters 5 and 8), both of which vary in fractions eluted from an ion-exchange column. Assessment of the influence of different adrenodoxin fractions by direct comparison of rates of a given steroid hydroxylation is therefore not practicable.

It was therefore decided to compare the ratio, ability of cholesterol side-chain cleavage:ability of 11β-hydroxylation, of successive adrenodoxin fractions eluted from a DEAE-cellulose column. Typically the adrenodoxin eluate was divided into five successive fractions. Ten "normal" incubation mixtures were set up containing equal amounts of adrenodoxin-free cytochrome P450 and flavoprotein in excess. Five flasks contained [4-^{14}C] cholesterol as substrate and the other five flasks contained DOC. From each of the five adrenodoxin fractions an equal aliquot was added to one pair of flasks containing [4-^{14}C] cholesterol or DOC. An attempt was made to keep the concentration / of adrenodoxin to a level where it would be the limiting factor and to see that the adrenodoxin concentrations of different pairs of flasks were equal. Fig. 27 shows the results obtained for four such pairs, the fifth pair of this experiment having been rejected during the experiment. Although the absolute rate of conversion of [4-^{14}C] cholesterol was low in these experiments it would appear that the ability to 11β-hydroxylate is more marked with adrenodoxin eluted at lower salt concentration and the relative abilities tend to be reversed till the cholesterol side-

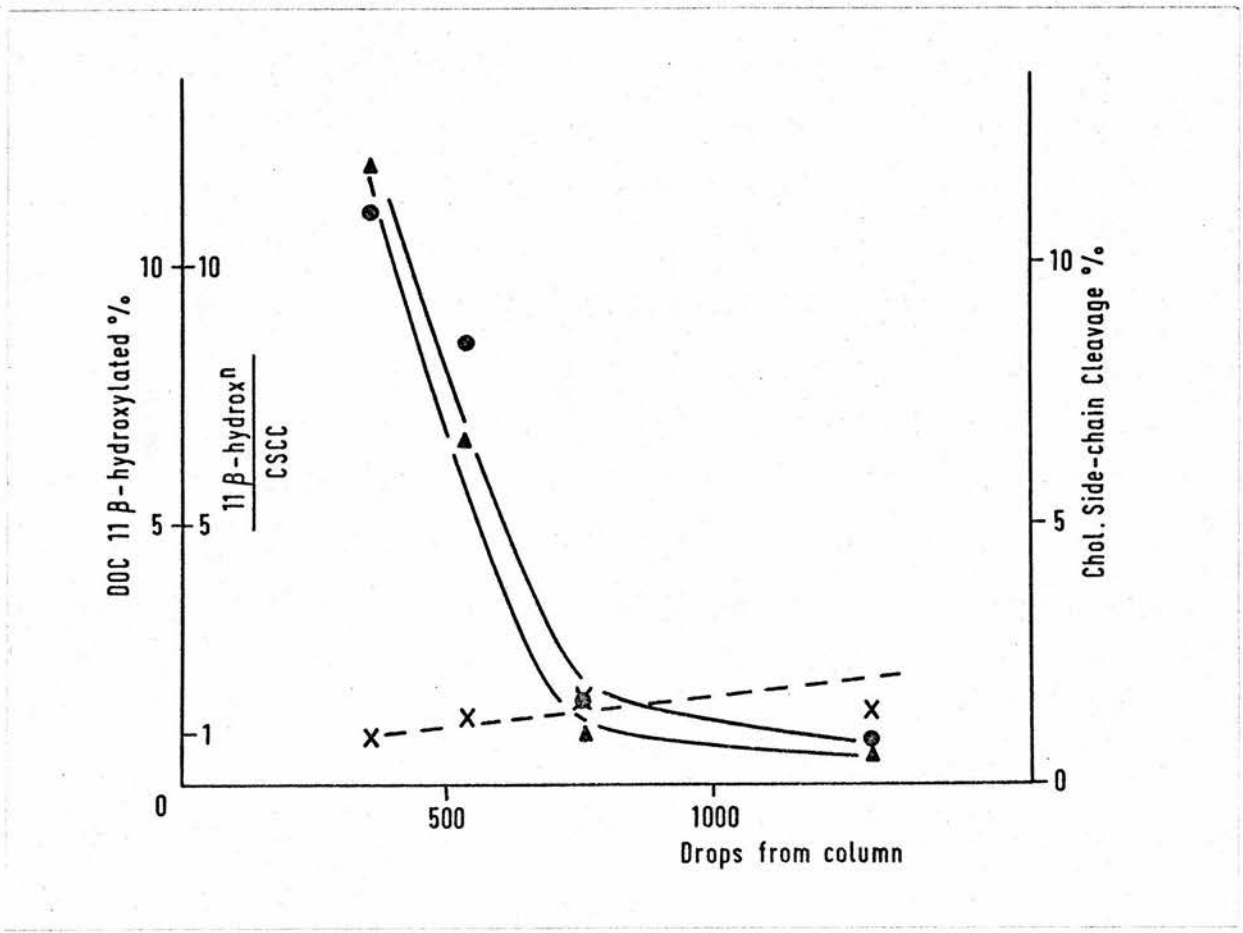


Fig. 27 Effect of different adrenodoxin fractions from DEAE-cellulose chromatography on reconstitution of steroid hydroxylases

× ——— × Cholesterol side-chain cleavage activity

● ——— ● DOC 11 β-hydroxylation activity

▲ ——— ▲ DOC 11 β-hydroxylation activity:cholesterol side-chain cleavage activity (CSCC)

Experimental details as described in the text.

chain cleavage activity is more important using adrenodoxin eluted at higher salt concentrations. The above work was not decisive on the complex question as to the existence of isoenzymes of adrenodoxin and perhaps a different approach to separation or evaluation may yield more conclusive evidence as to their presence or absence. The significance of the amino acid composition of adrenodoxin in this connection is referred to later in this chapter. If several forms of adrenodoxin are present and all transfer reducing equivalents equally well to the haemprotein, cytochrome c, it may be that their differences lie in their primary or secondary structure or in their interaction with the flavoprotein.

Molecular weight determination

The molecular weight determination was carried out by gel-filtration on Sephadex G200. (Kimura and Suzuki, 1967, had reported obtaining a value of about 15,000 by gel-filtration but details of the column and experimental procedure used were not specified.) Sephadex G200 which had been allowed to swell in Tris buffer for several weeks was poured into a column. The final bed size was 95 cm x 2.7 cm diameter. Several volumes of Tris buffer from a head of 12 cm were passed at a flow rate of 14.5 ml/hour through this over two weeks to stabilise the gel. Markers successfully used to calibrate the gel included bovine fibrinogen of true mol.wt. 330,000 but apparent mol.wt. by gel-filtration 733,000, and ovalbumin of true mol.wt. 45,000 but apparent mol.wt. by gel-filtration 41,000 (Andrews, 1965). Blue dextran and ε-DNP-L-lysine were generous gifts from Dr. J. Kay, pepsinogen from Dr. A. Ryle and ovalbumin, bovine serum albumin and apoferritin from Dr. D. K. Apps. Bovine fibrinogen (BDH) was previously prepared for use according to Laki (1951). Calibration of the column was performed over several different runs

and most of the markers were located by their spectral extinction at characteristic wavelengths. Yeast glucose-6-phosphate dehydrogenase was located according to Kornberg and Horecker (1955) and its chromatographic behaviour corresponded to a mol.wt. of about 128,000.(Andrews, 1965). Pepsinogen was assayed by acid digest of haemoglobin.

Fig. 28 shows the calibration curve obtained for this column. Adrenodoxin was found to chromatograph in the same region as cytochrome c. Assuming that adrenodoxin has the chromatographic properties of an "ideal" protein this would place its molecular weight at around 12,400.

Electrofocussing behaviour (see Methods and Chapter 3)

The isoelectric point of adrenodoxin is one of its characteristics which have not yet been reported. Furthermore the miniature electrofocussing technique described by Koch and Bachx (1969) can be used to separate and demonstrate isoenzymes using a small amount of protein preparation. A sample of adrenodoxin was therefore examined by the miniature electrofocussing technique. However the high content of glutamic and aspartic residues in adrenodoxin result in its migration to the acid region of the pH gradient where the iron-sulphur chromophore is destroyed and the protein denatured. Two regions of dense and light denatured protein were observed at about pH 5.0 (Fig. 29) on completion of the run.

Amino acid analysis

Amino acid compositions of bovine adrenodoxin have been published by Kimura's group (Kimura, 1968; Kimura et al, 1969) and by Yasunobu's group (Tanaka, Haniu and Yasunobu, 1970). Owing to certain discrepancies between the two reports it was decided to make an independent assessment of the amino acid composition of bovine adrenodoxin. A sample of protein ($E^{415nm}:E^{280nm}$ = 0.80) was dialysed overnight against

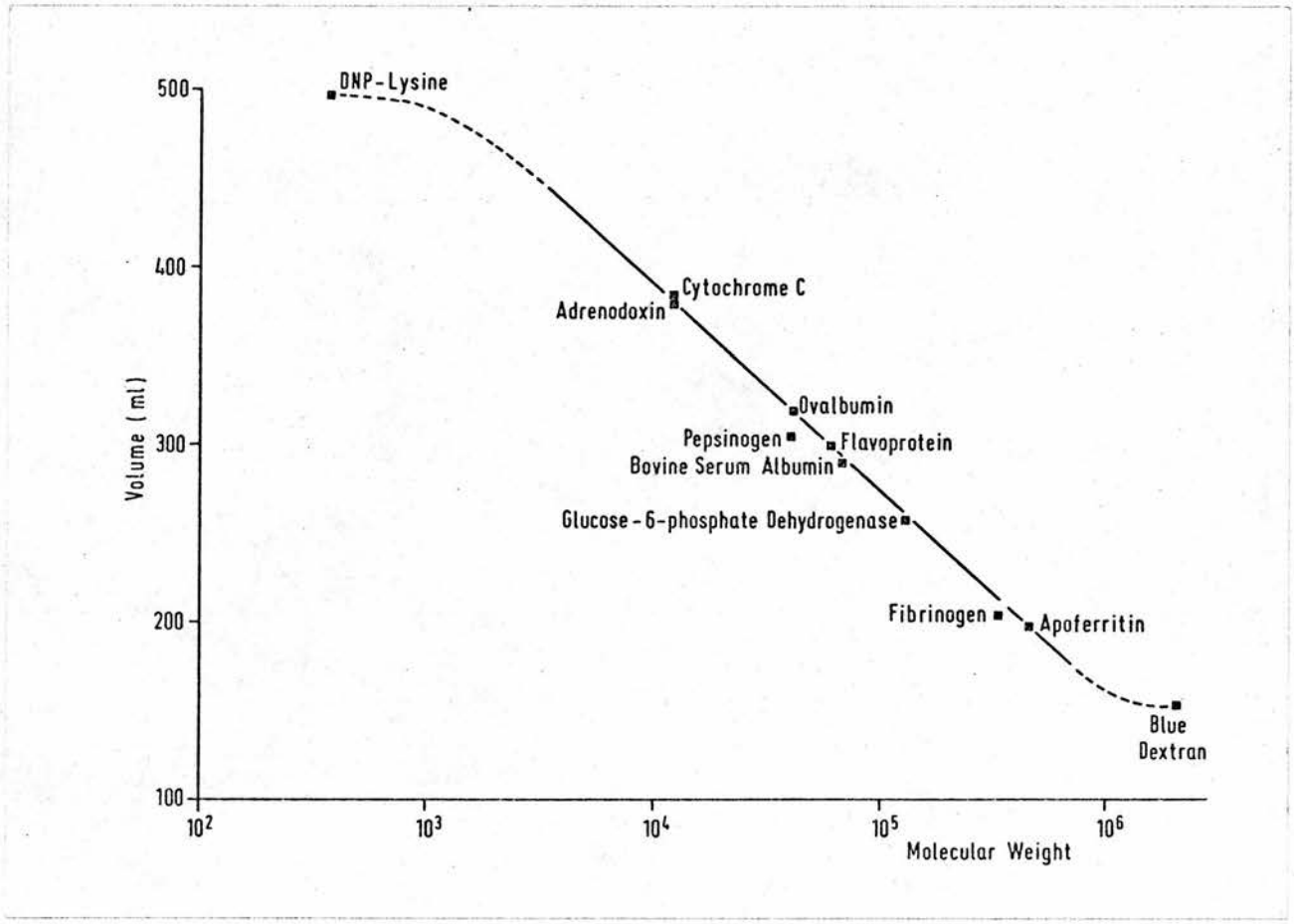


Fig. 28 Determination of the molecular weight of adrenodoxin by gel-filtration

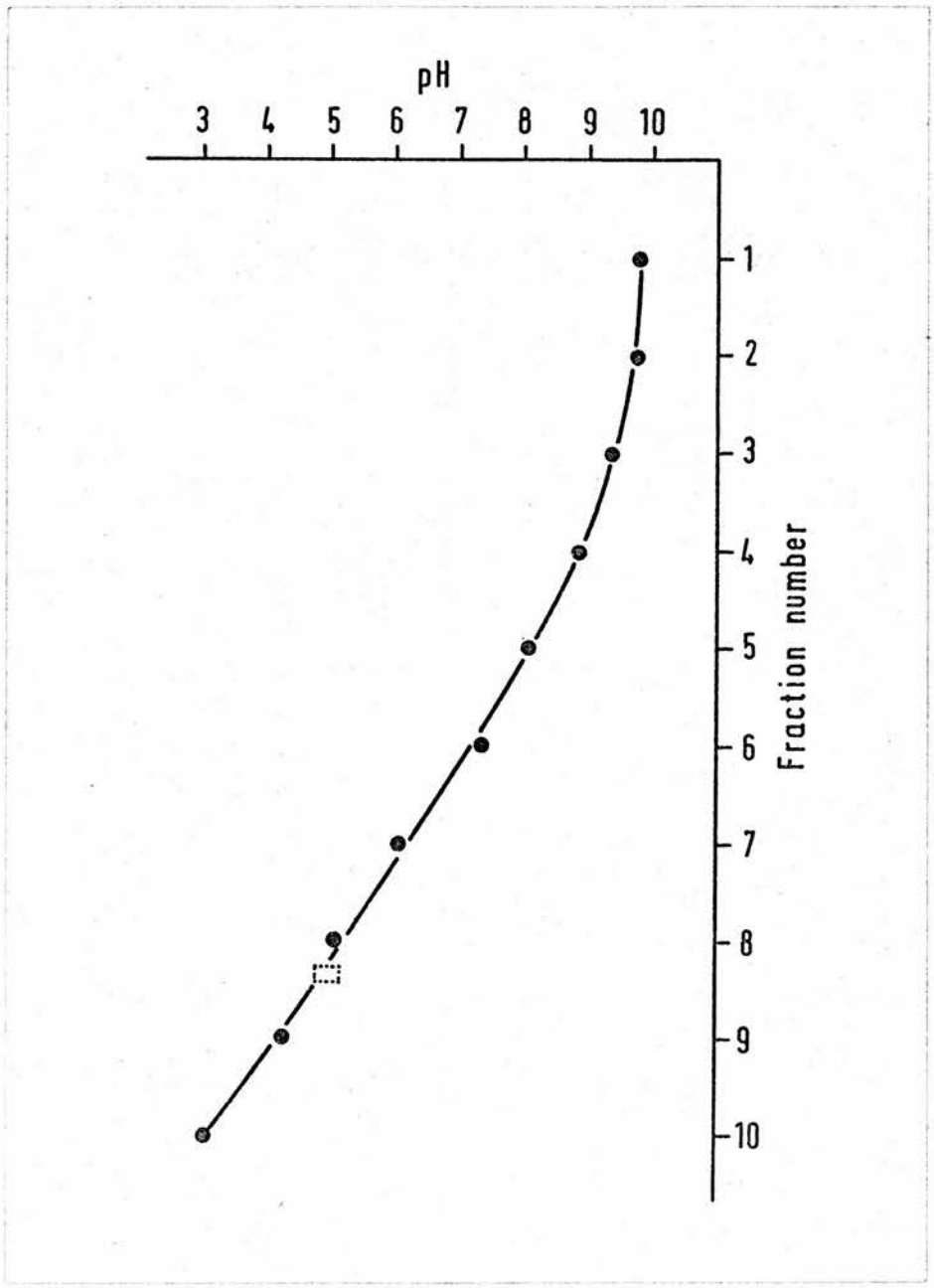


Fig. 29 The isoelectric point of adrenodoxin by miniature electrofocussing

The location of the denatured protein is indicated by the small rectangle.

distilled water and hydrolysed in 6 N-HCl at 105°C for 24 hours. The composition is noted (Table VI) with previously published figures for comparison.

From the table it is seen that there is close correspondence in values for the large majority of amino acid species in the three analyses especially in view of the larger number of residues allowed for per molecule in the Yasunobu data. This also indicates the comparable state of purity of the three preparations. An important and interesting difference between the three analyses is the contents of "aspartic" and "glutamic" residues. There is a disparity in the absolute values obtained for these residues between the three preparations and in the present work glutamic residues considerably exceed the aspartic while the reverse applies in the case of the other two preparations. The possible significance of this is discussed in Chapter 9.

Summary

1. Experience acquired during the present work on methods of purification of adrenodoxin is described together with the outline scheme of purification presently in use.
2. Methods of determining the progress of purification are discussed. The best preparations obtained in this work had $E^{415nm}:E^{280nm}$ = 0.80-0.81. Methods of determining the protein content of purified adrenodoxin preparations are compared. The nitrogen content of adrenodoxin is given.
3. Investigations which have been made into the possible existence of several forms of bovine adrenodoxin are described.
4. Gel-filtration studies showed adrenodoxin to have a molecular weight of about 12,000.

5. Miniature electrofocussing resulted in the denatured protein coming out of solution at about pH 5.0.

6. The amino acid analysis is very close to previously published figures except in the quantities and relative proportions of glutamic and aspartic residues.

Table VI Amino acid composition of adrenodoxin

Residue	Nearest integer this work	Kimura's group	Yasunobu's group
Asx	11	14	19
Thr	6	7	10
Ser	6	6	7
Glx	15	10	11
Pro	0	1	1
Gly	7	7	8
Ala	6	6	7
Val	8	5	7
Met	2	1 or 4	3
Ile	6	7	8
Leu	11	9	12
Tyr	1	1 or 2	1
Phe	3	3	4
His	2	3	3
Lys	6	5	5
Arg	3	3	4
Trp	n.d.	0	0
Cys($\frac{1}{2}$)	n.d.(4?)	4	5
Total residues	97	94	115

(n.d. = not specifically determined)

CHAPTER 5
RESULTS AND EXPERIMENTAL

RECONSTITUTION OF THE ELECTRON-TRANSPORT CHAIN

The steroid hydroxylating system of adrenal mitochondria consists of a flavoprotein, adrenodoxin and cytochrome P450. Since the hydroxylation of a steroid molecule requires the supply of two reducing equivalents one would expect a simple stoichiometry of the three proteins such as 1:1:1 or 1:2:1 in the intact system depending on factors such as the number of electrons carried by each protein.

Despite earlier reports by Kimura and Suzuki (1967) and Kimura et al. (1967) that adrenodoxin requires 2 electrons per molecule for complete reduction as determined by potentiometric titration, more recent work by Orme-Johnson and Beinert (1969c) using combinations of anaerobic titrations with solid dithionite together with EPR and optical spectroscopy indicated the one electron per molecule full reduction of bovine and pig adrenodoxins. ~~In the course of the present work the apparatus devised by Foust et al. (1969) and used for~~ titrating other iron-sulphur proteins by Mayhew et al. (1969) was used to investigate a preparation of adrenodoxin. It was confirmed that less than two electrons per molecule of adrenodoxin were sufficient to produce reduction of the visible chromophore. The slow reduction of protein-bound ferric ion by reduced adrenodoxin gives ~~rise to an initial lag-period as in some plant and bacterial ferredoxins (Mayhew et al, 1969).~~

Stoichiometry of reconstitution of steroid hydroxylase

Fig. 30 shows the results of a series of incubations in which the purified adrenodoxin concentration / was 11.04 μM and the cytochrome P450 concentration varied. It is seen that the maximal cholesterol side-chain cleavage was obtained at 0.31 μM-cytochrome P450 (being equal to [adrenodoxin]:[cytochrome P450]::36:1) with lesser conversions at higher concentrations of cytochrome P450.

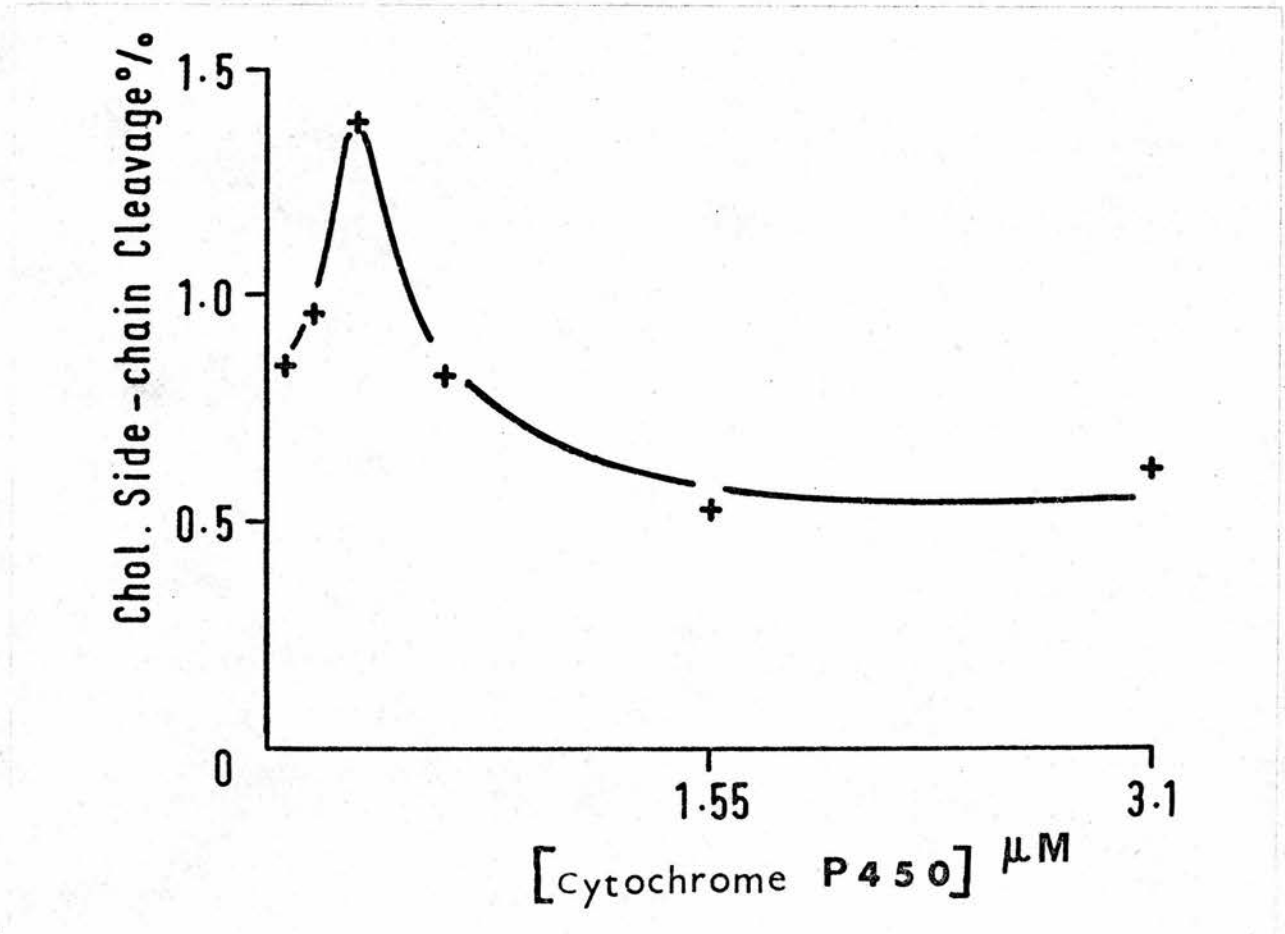


Fig. 30 Cholesterol side-chain cleavage activity at various cytochrome P450 concentrations

Effect of protein content on stoichiometry

The possible reasons for these surprising findings are discussed at greater length in Chapter 9 but Fig. 31 shows the effect of adding soluble non-catalytic protein in the form of bovine serum albumin to assays designed to examine the reconstitution requirements of the 11 β-hydroxylation system. It would appear from this form of presentation that the proportion of adrenodoxin to cytochrome P450 required to achieve a given rate of hydroxylation is less in the presence of a higher concentration of protein which is not however a component of the steroid hydroxylating system. Also in the absence of added albumin, [adrenodoxin]:[cytochrome P450] of between 37:1 and 75:1 showed maximal activity. However, in the presence of albumin the saturation of the system with respect to adrenodoxin has not been achieved at the highest levels.

Effect of ionic strength on stoichiometry

Fig. 32 shows by contrast the effect of ionic strength on reconstitution of the cholesterol side-chain cleavage system. The difference in ionic strength between these two series of incubations was 0.1 and 0.2 M-Tris buffer and the cytochrome P450 used was a preparation essentially free of the DOC 11 β-hydroxylating variety (Jefcoate, Hume and Boyd, 1970). The figures for conversion indicate that in 0.1 M-Tris buffer the [adrenodoxin]:[cytochrome P450] of about 70:1 is not yet saturating while in 0.2 M-Tris maximal activity is observed at a ratio of 60:1. The fact that adrenodoxin is not yet saturating at the high ratio of 70:1 in 0.1 M-Tris buffer may be partly due to the absence of DOC 11 β-hydroxylating cytochrome P450 which contributes to the quantity of cytochrome P450 as measured by the extinction at 450 nm of the reduced-CO complex.

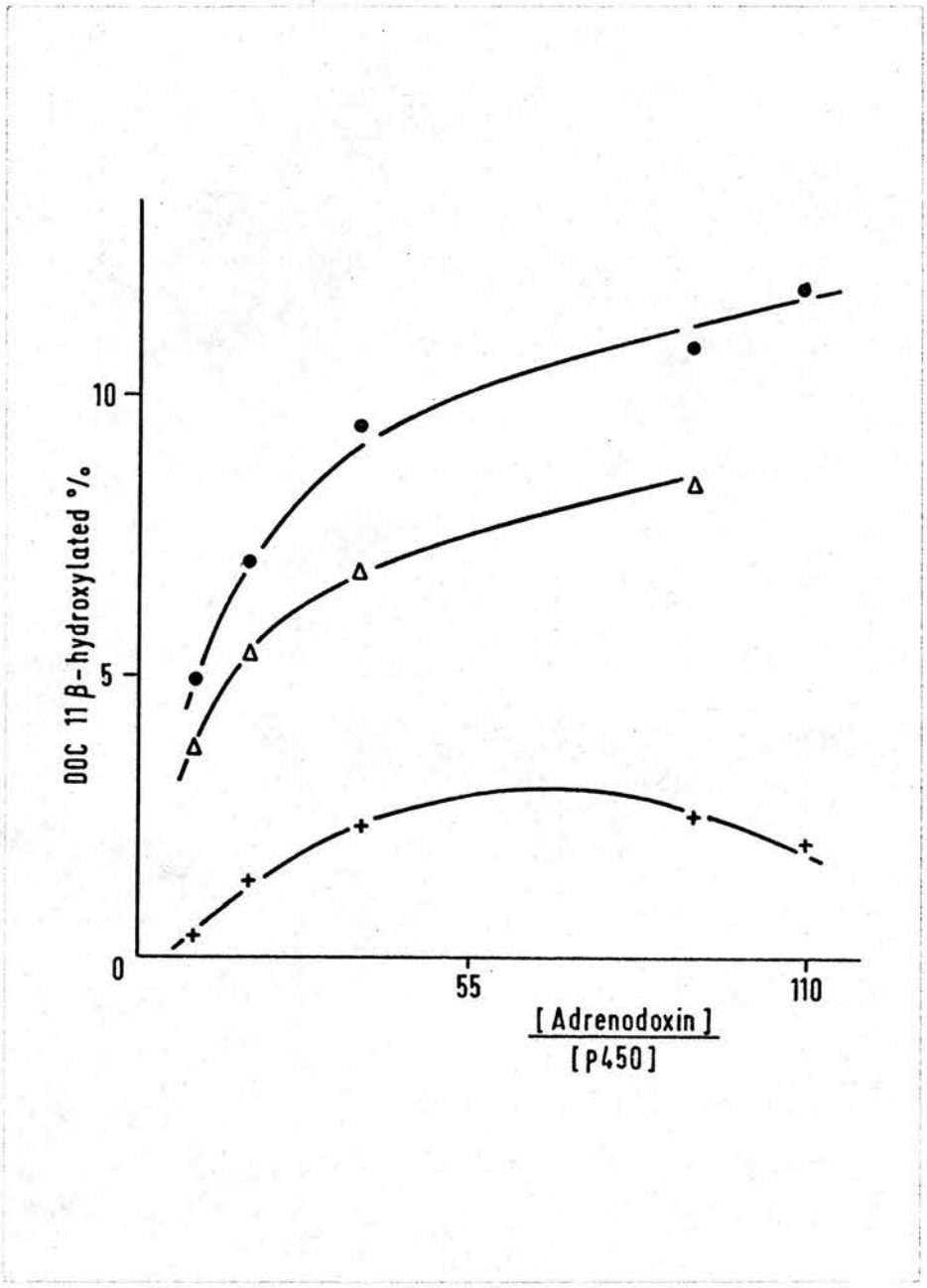


Fig. 31 The effect of bovine serum albumin on the activity of reconstituted DOC 11 β-hydroxylase

● ——— ● Bovine serum albumin (5.2 mg/ml) added

△ ——— △ Bovine serum albumin (19.1 mg/ml) added

\+ ——— \+ No bovine serum albumin added

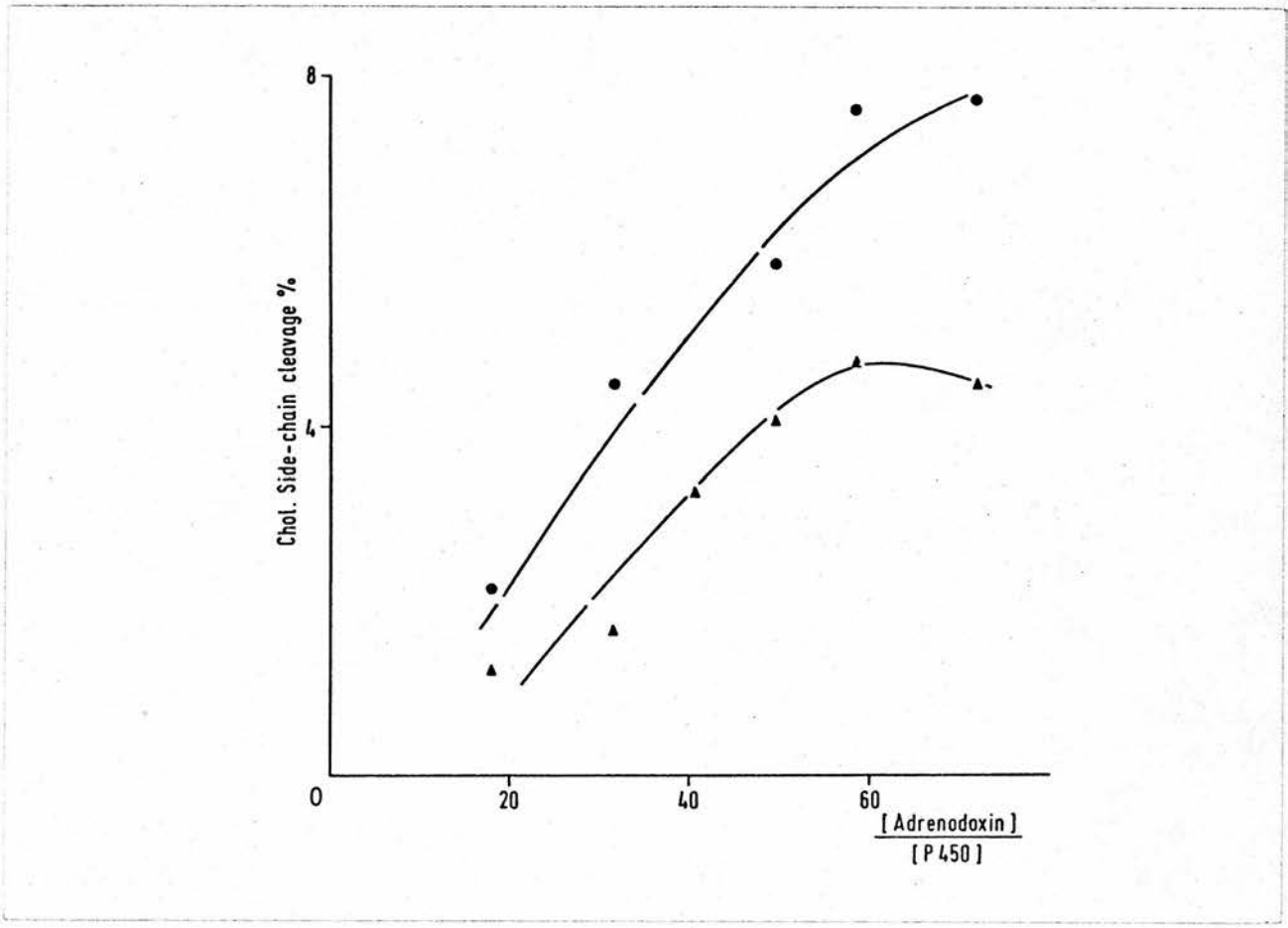


Fig. 32 The reconstitution of cholesterol side-chain cleavage activity at different ionic strengths

● ——— ● assays performed in 0.1 M-Tris-HCl, pH 7.4

▲ ——— ▲ assays performed in 0.2 M-Tris-HCl, pH 7.4

The above results show that salt concentrations, concentrations of proteins which are not hydroxylase components and active enzyme of different specificities may influence the high adrenodoxin requirements for *in vitro* reconstitution. Again excessive adrenodoxin appears to inhibit steroid hydroxylation.

Interaction of adrenodoxin and adrenodoxin reductase

Fig. 33 shows a titration of 1 ml adrenodoxin (1.38 mg/ml or 0.115 mM) with adrenodoxin reductase (8.6 mg protein/ml) in split cells using an Optika spectrophotometer. Addition of 43 µg of flavoprotein resulted in an increased transmission at about 360 nm which as shown changed rapidly in form on titrating in more flavoprotein. Both protein solutions were in distilled water. Fig. 34 shows that in a similar titration performed in 0.1 M-potassium phosphate buffer pH 7.4 about ten times the amount of flavoprotein had to be added to produce similar spectral changes. This difference could be attributed to the changed ionisation of the proteins in the buffer and (or) to the effect of the inorganic salt.

A similar titration of 1 ml adrenodoxin reductase (8.6 mg protein/ml) with adrenodoxin (2.76 mg/ml or 0.23 mM) showed comparable spectra in distilled water with interaction being marked after addition of 276 µg adrenodoxin onwards. The apparent extreme instability of the system may be the result of the interactions changing as the dielectric state of the medium changes as will be discussed later.

Effect of exogenous adrenodoxin on steroid hydroxylation by 100,000 g mitochondrial supernatant

Fig. 35 shows cholesterol side-chain cleavage by a 100,000 g x 45 min supernatant of solubilised fresh mitochondria in the presence and absence of exogenous adrenodoxin. The measurements were performed at different concentrations of NADPH and it is seen that addition of

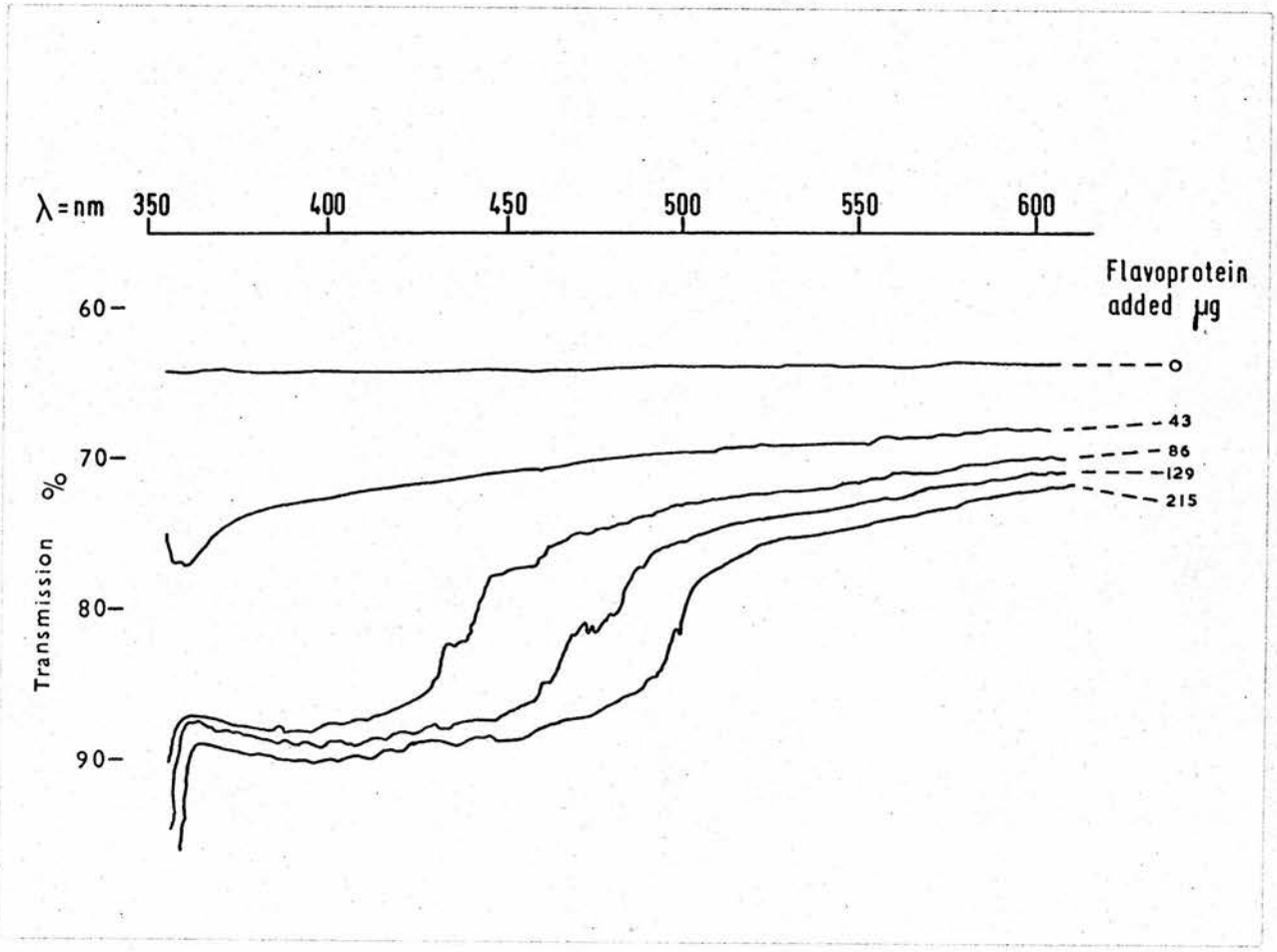


Fig. 33 The spectrophotometric titration of adrenodoxin with adrenodoxin reductase (in distilled water)

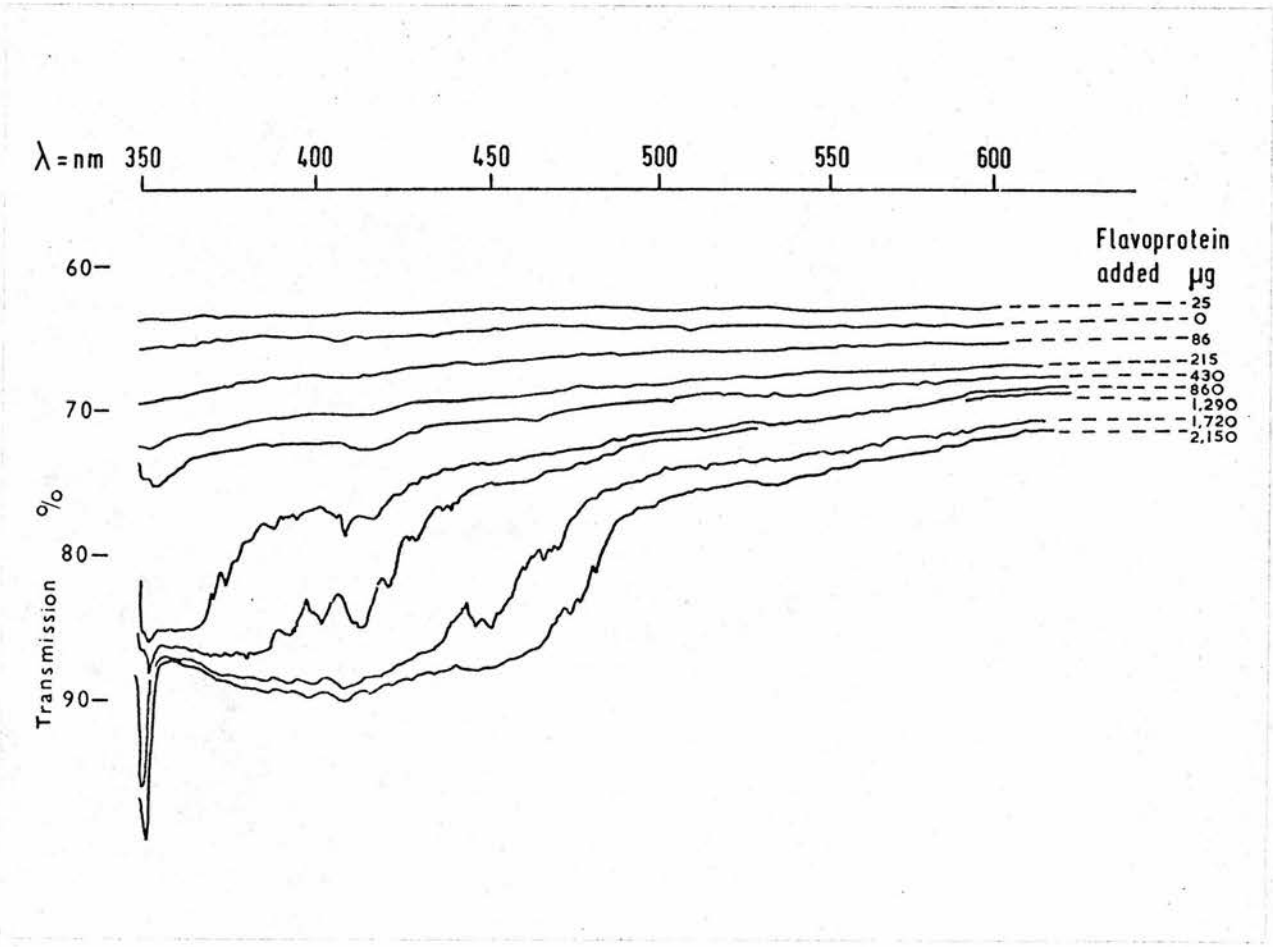


Fig. 34 The spectrophotometric titration of adrenodoxin with adrenodoxin reductase (in 0.1 M-potassium phosphate buffer, pH 7.4)

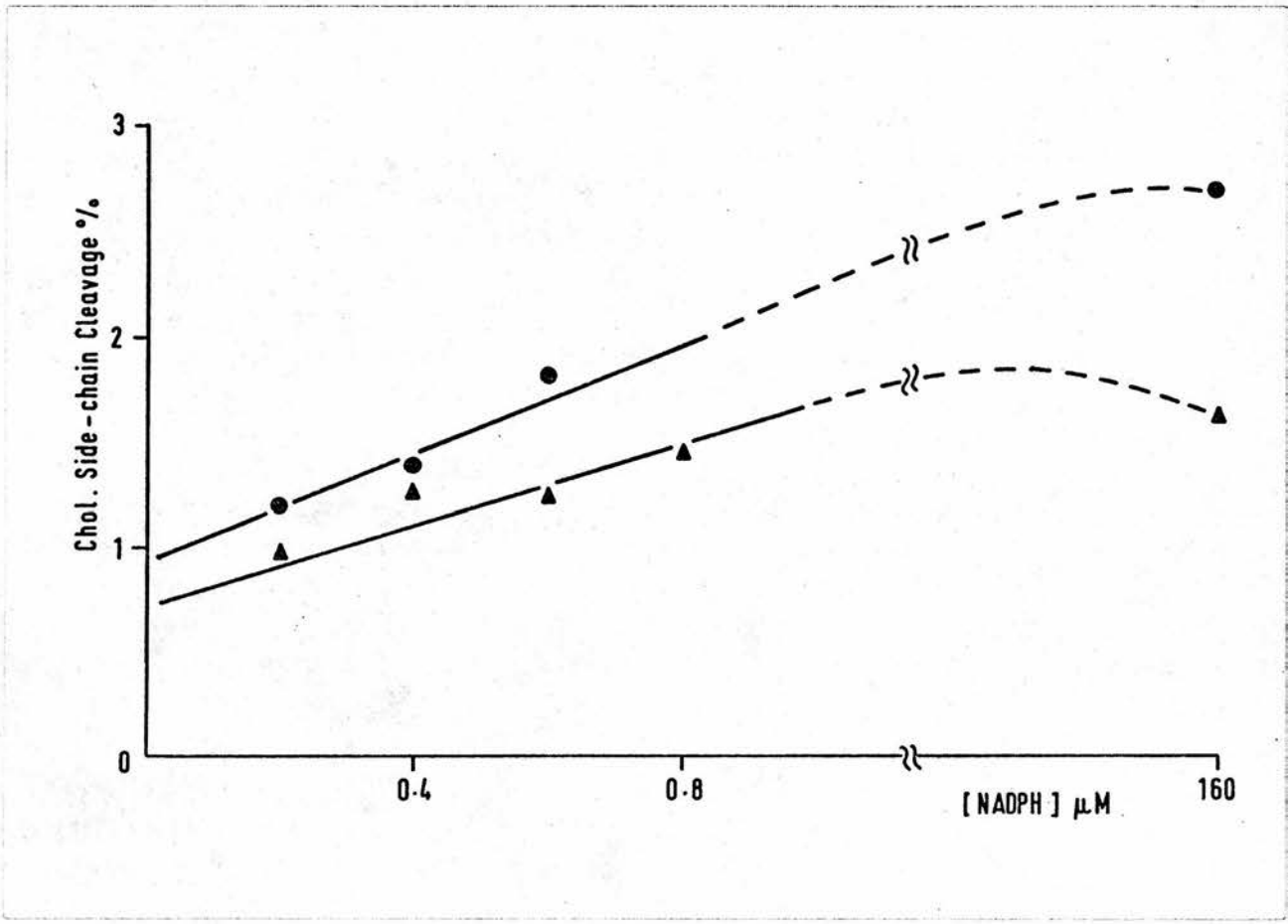


Fig. 35 The cholesterol side-chain cleavage activity of the mitochondrial supernatant in the presence and absence of exogenous adrenodoxin

● ——— ● Adrenodoxin (0.05 mM) added

▲ ——— ▲ No adrenodoxin added

adrenodoxin increases the rate of steroid hydroxylation. It is also seen that high concentrations of NADPH appear to cause inhibition of the enzyme system. Similar findings were found on investigation of the effect of exogenous adrenodoxin on the ability of the 100,000 g supernatant to 11 β-hydroxylate deoxycorticosterone.

Effect of excess adrenodoxin and NADPH on cytochrome c reduction

The reduction of cytochrome c by NADPH can be mediated by adrenodoxin plus adrenodoxin reductase (i.e. by mitochondrial cytochrome P450 reductase). Although the rate of reduction can be affected by ionic strength (Chapter 8) and other factors, under carefully controlled conditions the reaction can yield useful information. The effect of changing concentrations of NADPH and adrenodoxin were investigated. It was found that at low concentrations of adrenodoxin or NADPH excessive levels of the other factor (NADPH or adrenodoxin respectively) produced inhibition (Figs. 36 and 37).

Summary

1. The high proportions of adrenodoxin to cytochrome P450 required to achieve maximal activity of the steroid hydroxylating system *in vitro* have been confirmed. However an excess of adrenodoxin inhibits the rate of steroid hydroxylation.

2. Additions of bovine serum albumin, which is not a component of the steroid hydroxylases, appear to decrease the requirement of adrenodoxin to achieve high activities and increase that required to saturate the system. Increased ionic strength has the opposite effect.

3. The interaction of adrenodoxin with its reductase has been examined spectrally. The interaction appears to be complex and

adrenodoxin reductase adrenodoxin reductase
varies in appearance as / is titrated in. More / is required to achieve the same spectra in phosphate buffer than in distilled water.

4. It has been found that addition of exogenous adrenodoxin to a 100,000 *g* mitochondrial supernatant increases the rate *in vitro* of cholesterol side-chain cleavage and DOC 11 β-hydroxylation.

5. Excessive inputs of NADPH or adrenodoxin appear to inhibit the rate of cytochrome c reduction using adrenodoxin and its reductase.

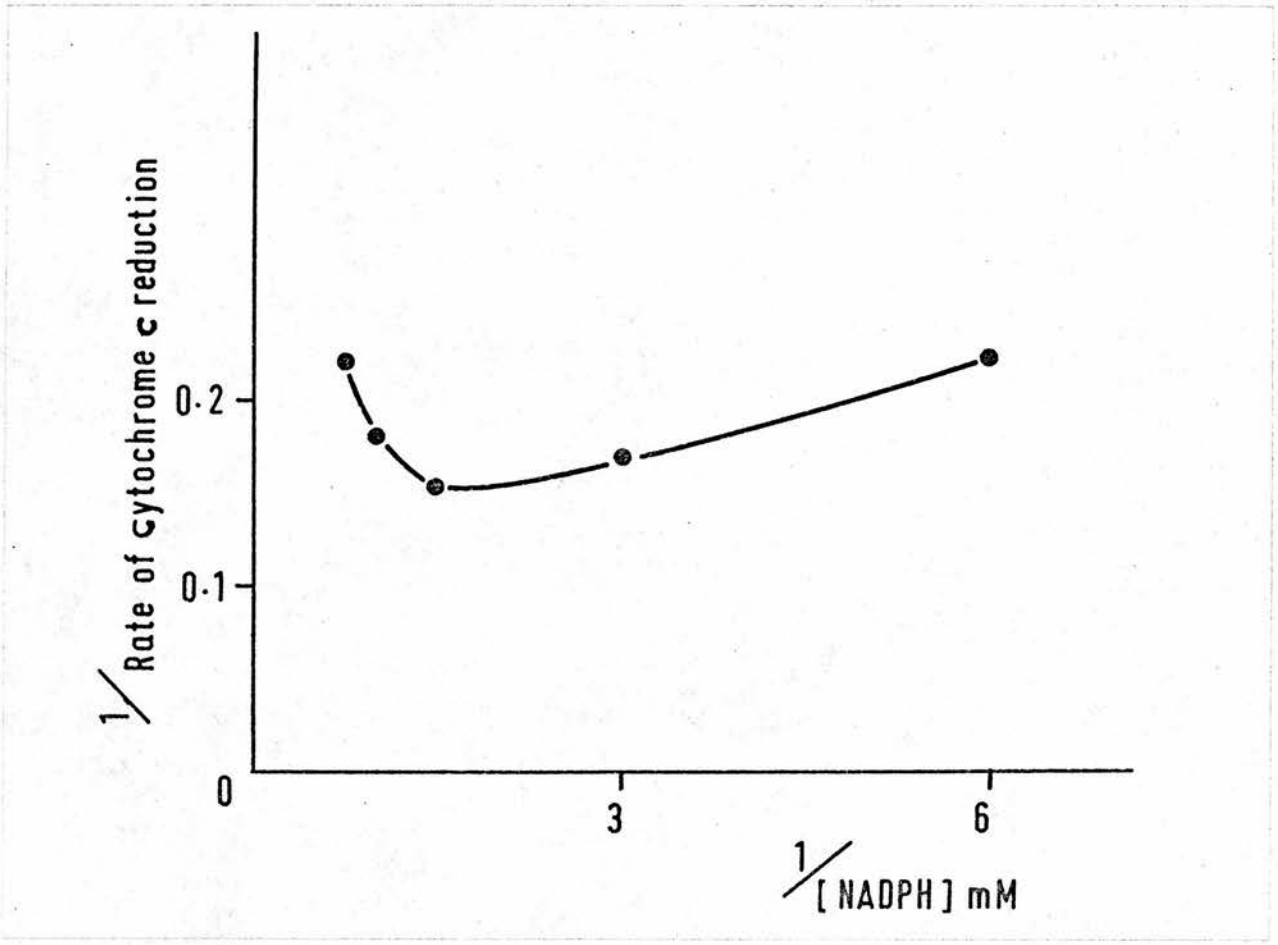


Fig. 36 The rate of cytochrome c reduction by adrenodoxin and adrenodoxin reductase at various concentrations of NADPH

The rate of cytochrome c reduction is measured as the rate of change of extinction at 550 nm per min at a fixed enzyme concentration of 25 µg/ml adrenodoxin reductase and 0.87 µM adrenodoxin.

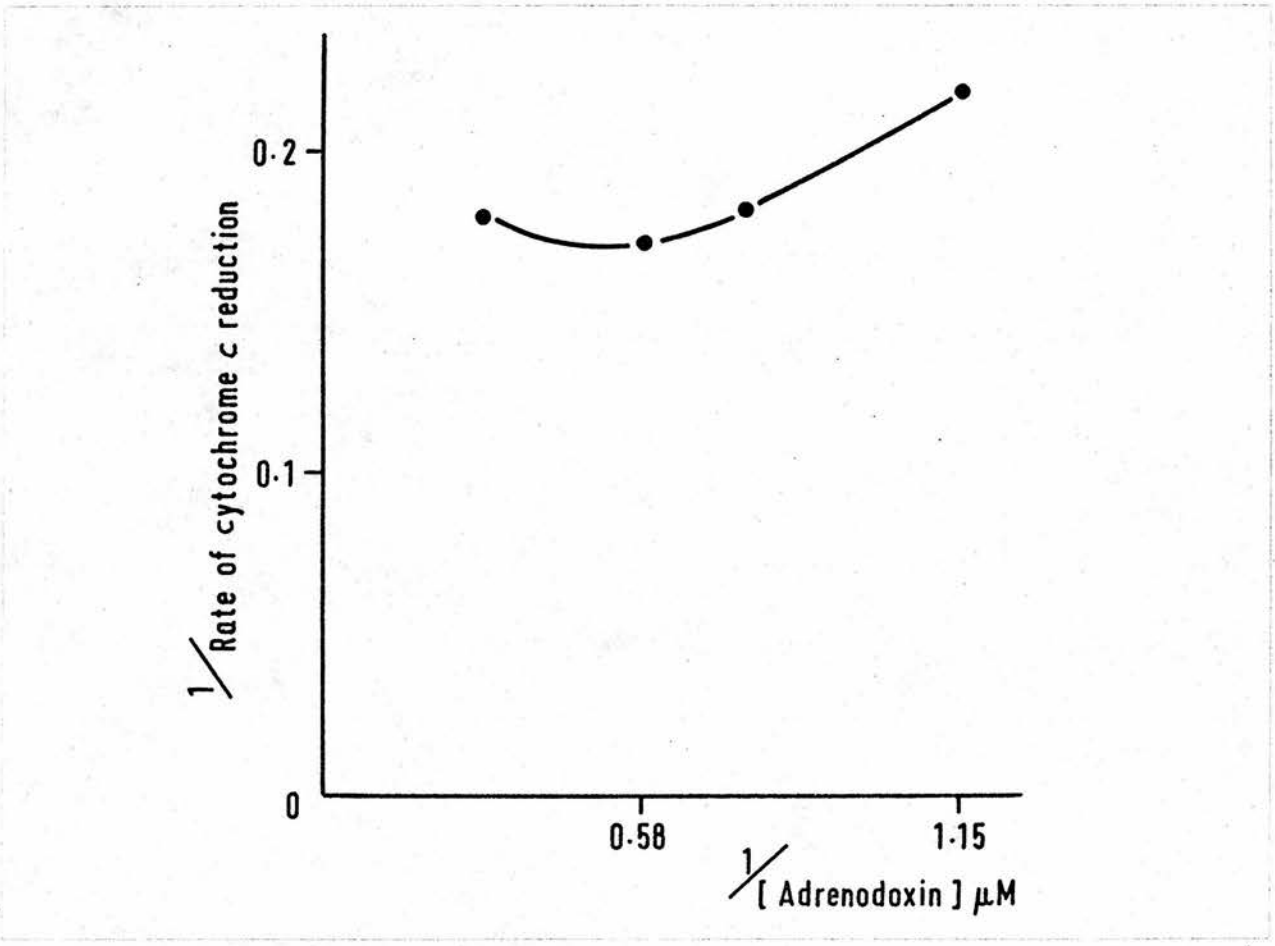


Fig. 37 The rate of cytochrome c reduction in the presence of adrenodoxin reductase and varying concentrations of adrenodoxin

The rate of cytochrome c reduction is measured as the rate of change of extinction at 550 nm per min at fixed concentrations of 25 µg/ml adrenodoxin reductase and 0.15 mM-NADPH.

CHAPTER 6
RESULTS AND EXPERIMENTAL

FACTORS INFLUENCING THE SUPPLY OF REDUCING EQUIVALENTS

Variation in the supply of reducing equivalents to adrenodoxin reductase is a possible means of affecting or controlling corticosteroidogenesis. The effect of varying the concentration of NADPH on corticosteroid hydroxylations and factors which could lead to this were therefore examined.

Km for NADPH for cholesterol side-chain cleavage and DOC 11 β-hydroxylation by sonicated mitochondrial enzyme

Fig. 38 shows the plot of the reciprocal of the DOC 11 β-hydroxylating activity as a function of the reciprocal of the NADPH concentration of the assay. The Km for NADPH for the DOC 11 β-hydroxylating activity was found to be 34.5 μM. The Km for NADPH with respect to the cholesterol side-chain cleavage activity (Fig.38a)was also investigated and found to be 2 μM, a figure which is in line with other observations (Mason, 1970) on placental side-chain cleavage.

Inhibition of DOC 11 β-hydroxylation by sonicated mitochondrial protein by excess NADPH

Fig. 39 shows that maximal DOC 11 β-hydroxylation is observed at NADPH concentrations of 0.15 mM with marked inhibition at higher levels of substrate. Tchen (1968) reported a similar inhibitory effect of excess NADPH on cholesterol side-chain cleavage with maximal activity at 0.05 mM. The inhibitory effect of excess NADPH on the reduction of cytochrome c mediated by adrenodoxin and adrenodoxin reductase has been noted (Chapter 5).

Investigation of adrenocortical NAD-kinase and NADP(H) levels

Although considerable data is available on the factors affecting supply of reducing equivalents for steroidogenesis, the (presumed) NAD-kinase of the bovine adrenal cortex did not appear to have been investigated in detail. Since this is a factor which could affect the supply of NADPH experiments were carried out on the bovine and rat adrenal NAD-kinases.

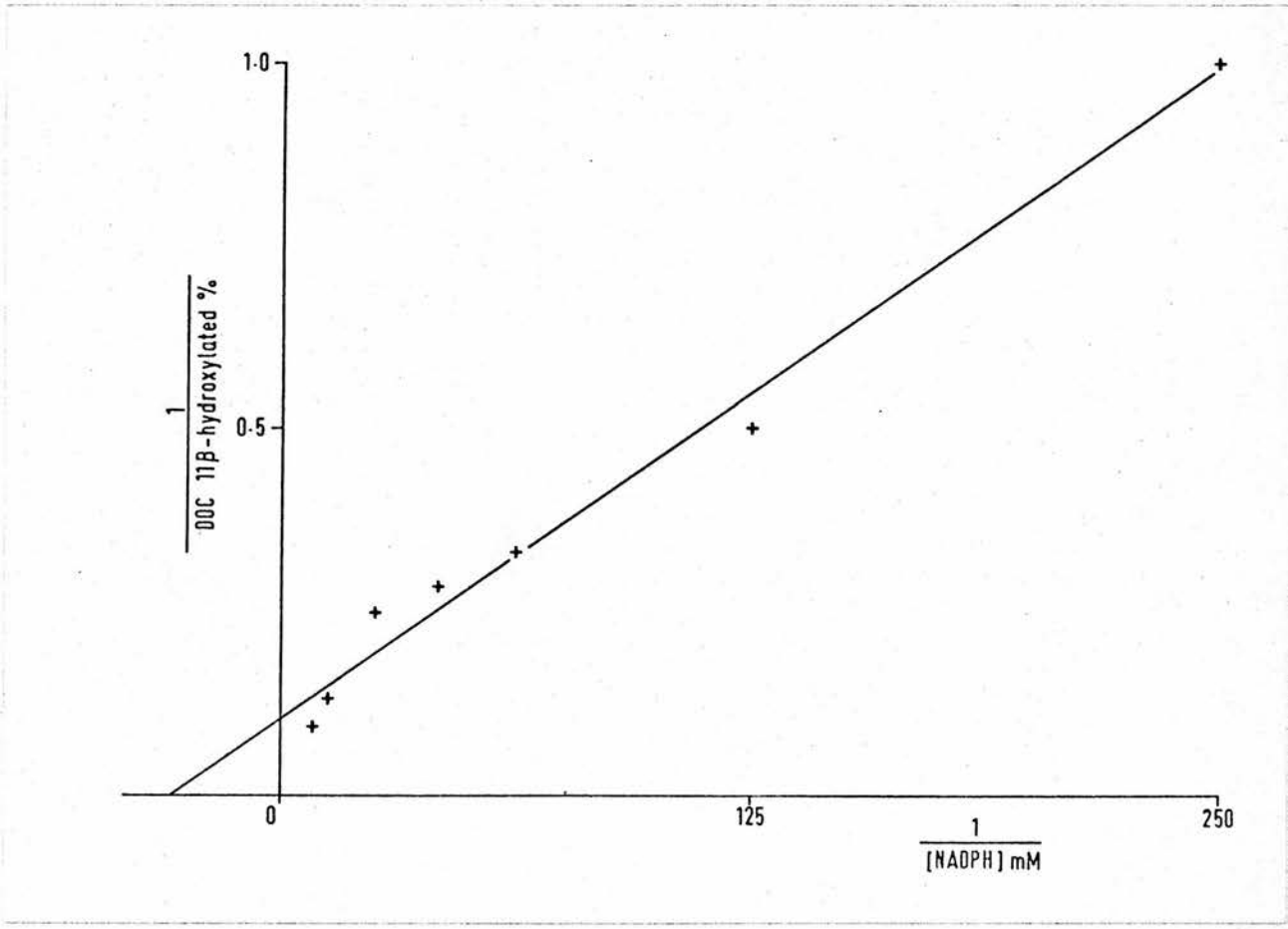


Fig. 38 The plot of the reciprocal of DOC 11 β-hydroxylating activity against the reciprocal of low concentrations of NADPH

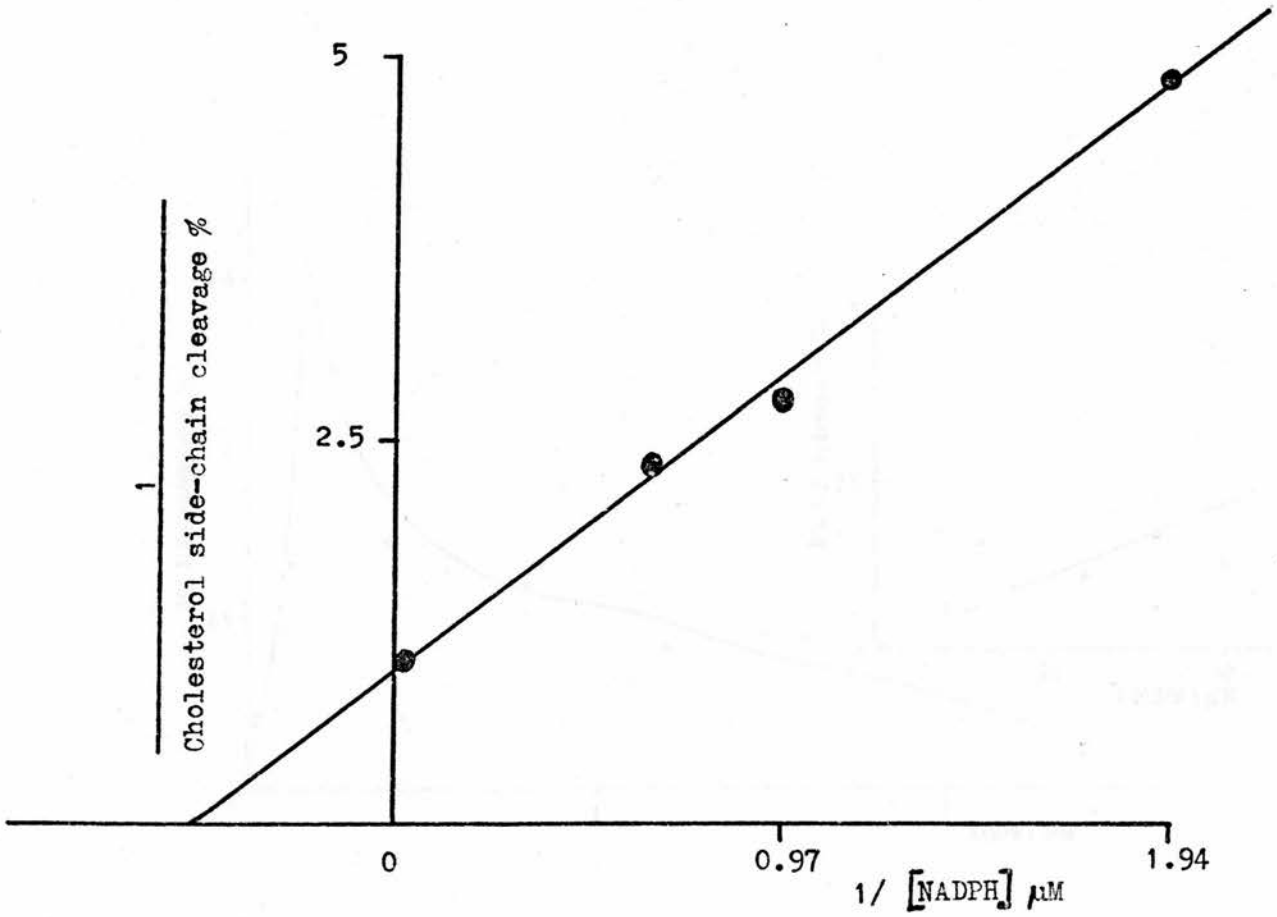


Fig. 38a The plot of the reciprocal of cholesterol side-chain cleavage activity against the reciprocal of low concentrations of NADPH

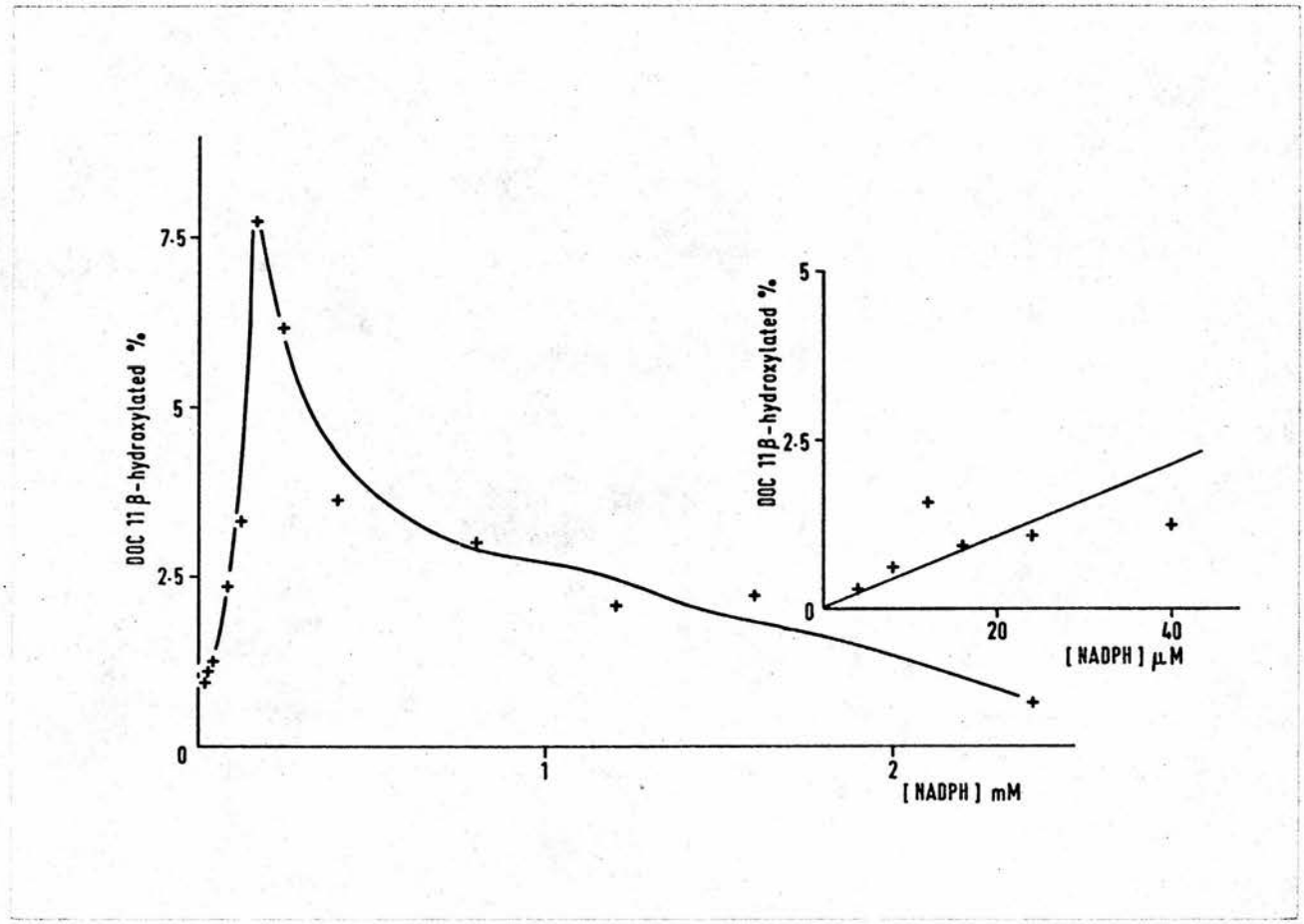


Fig. 39 The dependence of DOC 11 β-hydroxylating activity on the concentration of NADPH

(a) Localisation

Fresh bovine adrenocortical tissue was homogenised with a hand-operated Potter-Elvehjem homogeniser in 0.25 M-sucrose containing 5 mM-Tris and 1 mM-EDTA. The mitochondria, microsomes and cytoplasm were separated by centrifugation. The mitochondria and microsomes were washed twice in sucrose, resuspended in 20 mM-sodium bicarbonate and sonicated, while the cytoplasm (the supernatant of 100,000 g x 30 min centrifugation) was desalted on a Sephadex G25 column. The enzyme assays in 0.125 M-triethanolamine Cl, pH 7.3 buffer containing 15 mM-nicotinamide and determinations of NADP(H) levels were carried out as described by Apps (1970, 1971). The assay consists of the initial incubation of enzyme with NAD and ATP for 10 min at 30°C followed by the determination of the NADP(H) produced by measuring the reduction of DCPIP in the presence of phenazine methosulphate. Amounts down to 0.02 nmol NADP(H) can be accurately measured.

Table VII demonstrates clearly that the NAD-kinase content and highest specific activity reside largely in the cytoplasm. The specific activity is defined as nmol NADP(H) formed per mg protein per hour. The specific activity of the crude adrenocortical cytoplasmic protein of 5.2 nmol/mg protein/h is of the same order as crude protein specific activities from pigeon-liver cytoplasm (20) and rat whole liver homogenate (4).

A considerable amount of data obtained on the influence of NADP(H) levels on corticosteroidogenesis is discussed in McKerns (1968). Harding and Nelson's (1964) figures indicate that whole rat adrenal glands contain of the order of 0.3 nmol NADP(H) per mg tissue while Peron and McCarthy (1968) describe rat adrenal mitochondria as having 2.7 nM-NADP(H) per mg protein and microsomes 0.48 nM-NADP(H) per mg protein.

Table VII Subcellular localisation of NAD-kinase in bovine adrenal cortex cells

Cell fraction	Total protein recovered (mg)	Endogenous NADP(H) (nmol/mg protein)	Specific activity (S.A.) (nmol converted/ mg protein/h)	Enzyme recovered (S.A. x mg protein) (%)
Cytoplasm	373	-	5.2	1940 (95%)
Microsomes	26.1	0.63	0.80	21 (1.1%)
Mitochondria	88.2	0.5	0.93	82 (4.2%)

(b) Effect of ACTH and 3'-5' cyclic AMP on NAD-kinase of loose-cell preparations

ACTH and 3'-5' cyclic AMP have been shown to influence certain enzymes affecting NADPH levels in the adrenal cortex (Haynes and Berthet, 1957; Haynes, Sutherland and Rall, 1960; McKerns in McKerns, 1968 p.479). It was therefore of interest to see whether these agents could be shown to influence the NAD-kinase activity of this tissue.

For this investigation a loose-cell preparation of bovine adrenal cortex was used. The cortical tissue from twelve freshly collected adrenal glands was scraped into separate incubation flasks. To each was added 5 ml Hanks's medium (Parker, 1961) lacking glucose, calcium and phenol red but containing 0.5 mg/ml collagenase. The mixtures were incubated at 37°C for 60 min with shaking, the cells recovered by centrifugation and washed and resuspended in 10 ml of the complete Hanks's medium in incubation flasks. The twelve flasks were separated into three series being (a) controls - no additions, (b) plus 80 milliunits ACTH (Hog ACTH-zinc hydroxide Organon) per ml incubation, and (c) plus 3 mM-3'-5' cyclic AMP respectively. Incubations were carried out for 2 hours at 37°C. The cells were recovered by centrifugation, washed in 0.25 M-sucrose containing 10 mM-Tris, pH 8.0 and 5 mM-EDTA and homogenised (Ultra-Turrax, 20,000 rev./min for 5 seconds) in 0.1 M-Tris, pH 8.0 containing 1 mM-EDTA. The NAD-kinase was assayed and the NADP(H) formed measured by the polarographic method of Greenbaum, Clark and McLean (1965).

Table VIII shows that by this method of examination neither ACTH nor 3'-5' cyclic AMP were found to influence the level of NAD-kinase activity of the tissue. The slight decrease in NAD-kinase specific activity following treatment with ACTH and 3'-5' cyclic AMP is not

Table VIII Effect of ACTH or 3'-5' cyclic AMP on NAD-kinase levels

Cell preparation	No. of determinations	Specific activity ± standard deviation (nmol NADP(H)/mg protein/h)
Control	4	2.12 ± 0.57
plus ACTH (80 milliunits/ml)	4	1.51 ± 0.57
plus 3'-5' cyclic AMP (3 mM)	4	1.34 ± 0.46

significant and may be explained by the increased synthesis of other proteins in the tissue.

(c) Effects of ACTH in vivo on rat adrenal enzymes and NADP(H) levels

Six adult female rats were injected intraperitoneally with 2 units ACTH (Hog ACTH - Zinc hydroxide, Organon) in 0.2 ml of 0.9% NaCl. After 15 min they were killed by guillotine. Whole adrenals from pairs of animals (i.e. 4 glands per preparation) were homogenised in 1 ml of 0.125 M-triethanolamine buffer pH 7.3. Glands from six control (unstressed) adult female rats were treated similarly. Determinations were made of NADP(H) levels, NAD-kinase, NADPH-cytochrome c reductase and NADPH-dichlorophenolindophenol reduction activities. The two latter activities could provide information on cytochrome P450 reductase and adrenodoxin reductase activities and may be compared with studies reported by Kimura (1969). Averages of the values obtained (which were close to each other) are given in Table IX. It is seen that there was no significant increase in any of the factors examined following ACTH injection.

Investigation as to existence of histidine degrading enzymes

The degradation of histidine by histidase gives rise to urocanic acid. This is degradable to yield components of the tricarboxylic acid cycle (see Coote and Hassall, 1969) which can furnish reducing equivalents for steroid hydroxylations. In view of previous experience with histidine degrading enzymes, assay methods were available and it was decided to investigate bovine adrenal enzyme preparations to see if histidine degrading enzymes were present and if so to identify their subcellular location and assay their activity.

Bovine adrenal cortex tissue (about 14 gm) was homogenised in 15 ml 0.1 M-sodium phosphate buffer pH 7.4 first with a Waring Blender (top-drive, 30 s) and then with a glass homogeniser. The

Table IX Effect of ACTH injection in vivo on rat adrenal factors

Group	mg protein per ml homogenate	NAD-kinase (nmol NADP(H)/mg protein/h)	NADP(H) levels (nmol/mg protein)	cytochrome c reduction (Δ^{E550nm}/mg protein/min)	DCPIP reduction (Δ^{E590nm}/mg protein/min)
Control	42.5	8.6	0.19	5.42	24.5
ACTH-treated	42.0	8.4	0.19	5.54	19.9

homogenate was centrifuged at 600 g for 15 min. The pellet was discarded and the supernatant used for incubations after a brief sonication to complete the disruption of the mitochondria. Control incubations were carried out with protein which had been placed in a boiling water bath for 5 min. Incubations were carried out in the manner described previously (Wickramasinghe 1969, 1970; Wickramasinghe et al, 1967) in different conditions and deproteinised aliquots of the resulting supernatants paper-chromatographed (descending, in n-butanol-acetic acid-water::50:12:50 upper phase). Imidazoles resulting from alterations to the side-chain of histidine migrate faster in this solvent system than the parent amino acid and are located by spraying the chromatogram with diazotised p-chloroaniline.

Incubations typically contained histidine (0.4 M) -0.1 ml, α-ketoglutarate (neutral, 117 mg/ml) -0.1 ml, pyridoxal phosphate - 30 µg, enzyme and sodium phosphate buffer 0.1 M to 4 ml. Incubations were carried out with additions of both 7.9 mg and 39.5 mg protein per flask to allow the detection of high or low enzyme activities. They were carried out in the presence and absence of α-ketoglutarate to distinguish between possible transaminase and L-amino acid oxidase activities and at both pH 7.4 and pH 5.7 since some decarboxylases are more active at low pH. Incubations were initiated by the addition of histidine and carried out for 2 h at 37°C.

No indication was obtained as a result of these incubations of histidine with adrenal cortex protein of a significant diminution of the original / concentration of the amino acid or a conversion to other compounds containing the unsubstituted imidazole nucleus. This indicates the absence in the bovine adrenal cortex of histidase, histidine-α-ketoglutaric transaminase, L-amino acid oxidase and histidine decarboxylase. This suggests that histidine degradative

processes do not play a part in the supply or utilisation of reducing equivalents in the adrenal cortex. Similar negative results were obtained in parallel experiments with bovine adrenal medullary tissue so the findings are applicable to the entire gland.

Effect of oxidised and reduced glutathione on steroid hydroxylations

Oxidised and reduced glutathione have been shown to affect steroid hydroxylations in tissue other than the bovine adrenal gland. It has been suggested that the effect is due to an interference with the supply of reducing equivalents. Fig. 40 and 41 show the effect on DOC 11 β-hydroxylation by intact bovine adrenal mitochondria of adding oxidised and reduced glutathione respectively in final concentrations ranging from 1-20 mM. The results are very similar to those found by Sulimovici (1968) on cholesterol side-chain cleavage by intact mitochondria from immature ovaries from rats treated with Pregnant Mare Serum Gonadotrophin. In that instance as well the inhibition by oxidised glutathione was more marked than by reduced glutathione, which latter also slightly increased hydroxylation at the lowest concentration.

Summary

1. The Km for NADPH for the 11 β-hydroxylation of DOC by bovine adrenal cortex mitochondrial enzyme was found to be 34.5 μM while that for cholesterol side-chain cleavage was 2 μM.

2. The DOC 11 β-hydroxylation reaction was inhibited by excess NADPH, the maximal enzyme activity being obtained at an NADPH concentration of 0.15 mM.

3. Investigations were carried out on adrenal cortex NAD-kinase.

   (a) 95% of the NAD-kinase activity is located in the cytoplasm. The specific activity is 5.2 nmol/mg protein/h which is comparable to the activity of this enzyme in other tissues.

(b) the enzyme activity was not augmented on incubating bovine adrenal cortex loose-cell preparations with ACTH or 3'-5' cyclic AMP.

(c) NAD-kinase, NADPH-cytochrome c reductase and NADPH-DCPIP reductase activities of rat adrenal glands were not increased after ACTH injection *in vivo*.

(d) Examination was made of bovine and rat adrenal NADP(H) levels.

4. Investigation was made but no evidence found of the presence in bovine adrenal cortex of any enzymes which degrade histidine.

5. Oxidised and reduced glutathione (up to 20 mM) both inhibited 11 β-hydroxylation of DOC by intact adrenocortical mitochondria though ~~reduced~~ glutathione at low concentration (1 mM) slightly increased the activity.

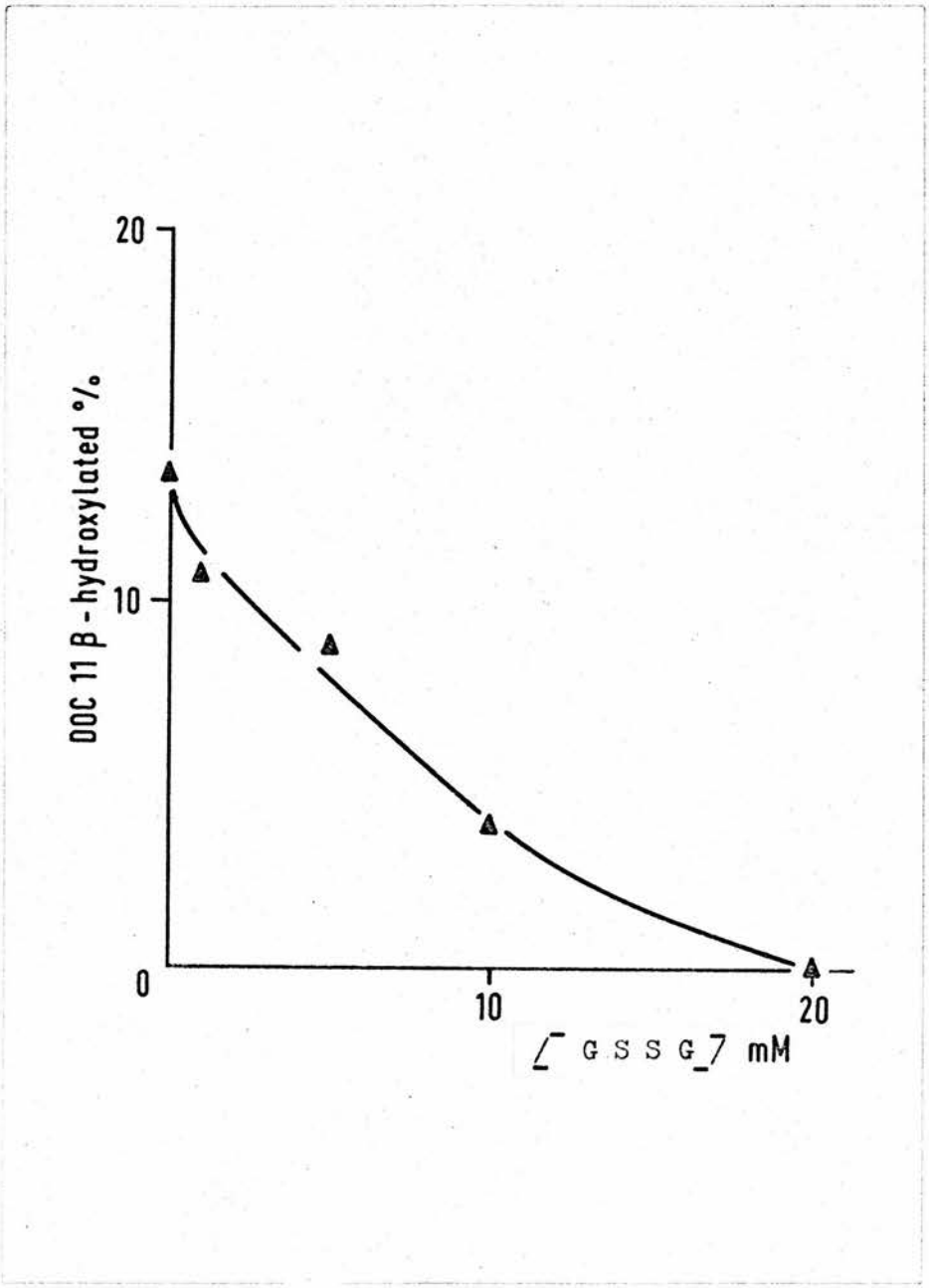


Fig. 40 The DOC 11 β-hydroxylating activity of intact adrenal cortex mitochondria in the presence of oxidised glutathione

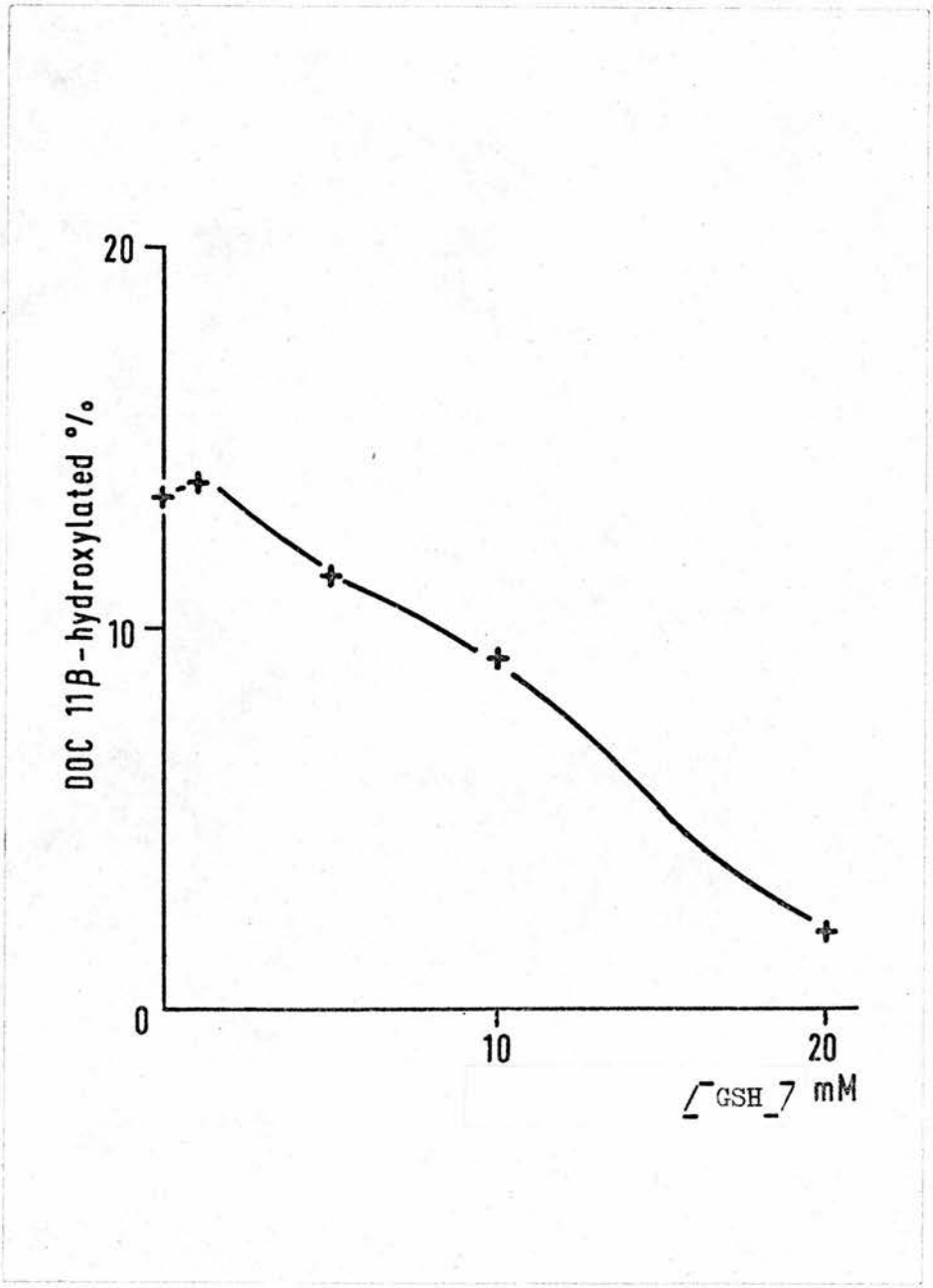


Fig. 41 The DOC 11β-hydroxylating activity of intact adrenal cortex mitochondria in the presence of ~~reduced~~ glutathione

CHAPTER 7
RESULTS AND EXPERIMENTAL

AN INTERACTION BETWEEN PHOSPHOLIPIDS AND THE PROTEINS OF THE STEROID HYDROXYLATING SYSTEM

It was decided to extend the work of Das and Crane (1964) on the complexing of cytochrome c with phospholipid to form a proteolipid to see whether adrenodoxin and cytochrome P450 behave in a similar manner. It had been found that cytochrome c interacts with several phospholipids to form distinct complexes. These complexes were soluble in isooctane to give a light extinction spectrum identical with that of cytochrome c in aqueous solution. Oxidised or reduced cytochrome c formed complexes and an isooctane solution of a complex containing oxidised cytochrome c yielded on addition of dithionite the spectral changes characteristic of cytochrome c reduction. It was decided to examine the possible formation of isooctane-soluble complexes of phospholipid with adrenodoxin and adrenal mitochondrial cytochrome P450.

Experiments were first carried out with cytochrome c and phosphatidylethanolamine as described by Das and Crane (1964). Initial attempts using ethanol as a dehydrating agent to accelerate complex formation were not very successful although a sample using reduced cytochrome c showed some "haemprotein extinction" in the isooctane layer. However later attempts using acetone instead of ethanol were very successful, the isooctane layer giving the characteristic cytochrome c extinction spectrum.

Evidence for the interaction of phospholipid with adrenodoxin and its stoichiometry

Some (impure) lecithin and/(impure) phosphatidylethanolamine were sonicated in distilled water for 10 min without interruption (in the cold room but without additional cooling). This treatment gave a satisfactory suspension of phospholipid which, after dialysis for 8 hours against 1,000 vol.cold distilled water (with one change), was ready for use after a further brief sonication. A solution of adrenodoxin (E^{415nm} = 0.7) was dialysed against 400 vol.cold distilled water for 4 hours before use.

Various dilutions and combinations of protein (1.6 ml) and phospholipid (0.5 ml) were investigated in conjunction with 0.9 ml redistilled acetone and 3 ml isooctane. The mixtures were placed in glass-stoppered tubes which were clamped horizontally on a reciprocal shaker and shaken for 15 min at about 200 strokes per min in the direction of the long axis. The tubes were then centrifuged at 1,500 rev./min for 5 min or until complete separation of the phases occurred. In the absence of salts in the dialysed solutions no phospholipid is extracted into the isooctane phase (Das and Crane, 1964). This was confirmed even after shaking overnight after addition of a little solid dithionite although some neutral lipid was extracted into the isooctane phase (see Fig. 44).

Material insoluble in both isooctane and aqueous phases collects at the interface but the extinction spectrum in the visible range characteristic of the iron-sulphur chromophore of adrenodoxin was not found in the isooctane phase after any of the treatments carried out. Three possible explanations for this could be (a) adrenodoxin does not form isooctane-soluble proteolipid, (b) the isooctane-soluble proteolipid formed from adrenodoxin does not have the same optical spectrum as an aqueous solution of the native protein or (c) the adrenodoxin chromophore which is much more labile than the chromophore of cytochrome c is destroyed by the harsh treatment used in preparing the complex. The isooctane phase was therefore examined spectrally over the ultraviolet range of the spectrum where a marked extinction at 325 nm was noticed. Although the presence of acetone in the isooctane phase interfered with examination of the "protein extinction" at around 276 nm, extinction at 325 nm is a characteristic of aqueous solutions of adrenodoxin (Kimura and Huang, 1970).

Duplicate one ml specimens of the isooctane solution prepared as

described above were dried down and the total phosphate content determined using amidol-bisulphite and ammonium molybdate as described in "Methods". Duplicate isooctane blanks were also assayed and found to contain no phosphorus while the duplicate one ml samples contained 0.4 and 0.36 μg-atom phosphorus each. This gave a value of about 4 g-atom of phosphorus in the form of phospholipid which combines with 1 mole of adrenodoxin to give the isooctane-soluble complex. This is much lower than the 22-32 g-atom of phosphorus in the form of mixed phospholipids which complex with 1 mole cytochrome c (Das and Crane, 1964). It has however been proposed that the primary formation of the phospholipid-cytochrome c complex principally entails interaction between the negatively charged phospholipids and the positive free amino groups of the lysine and arginine of cytochrome c. Since, unlike cytochrome c, adrenodoxin is an acidic protein (Chapter 4), the small number of phospholipid molecules which bind with it is not surprising.

The nature of the phospholipids interacting with adrenodoxin was investigated by thin-layer chromatography of one ml aliquots of the isooctane solution on silica-gel G-coated glass plates (Das and Crane, 1964), the solvent system being chloroform:methanol:water::65:25:4. Fig. 42 shows that several phospholipids were present including phosphatidylethanolamine but lecithin was the largest single phospholipid component. Some neutral lipid extracted directly from the impure phospholipid into the isooctane is seen at the top of the plate.

<u>The interaction of phospholid with cytochrome P450</u>

The possible interaction of phospholipid with cytochrome P450 was examined. Adrenal mitochondria were sonicated and washed free of adrenodoxin by repeated suspension in 0.154 M-KCl and sedimentation

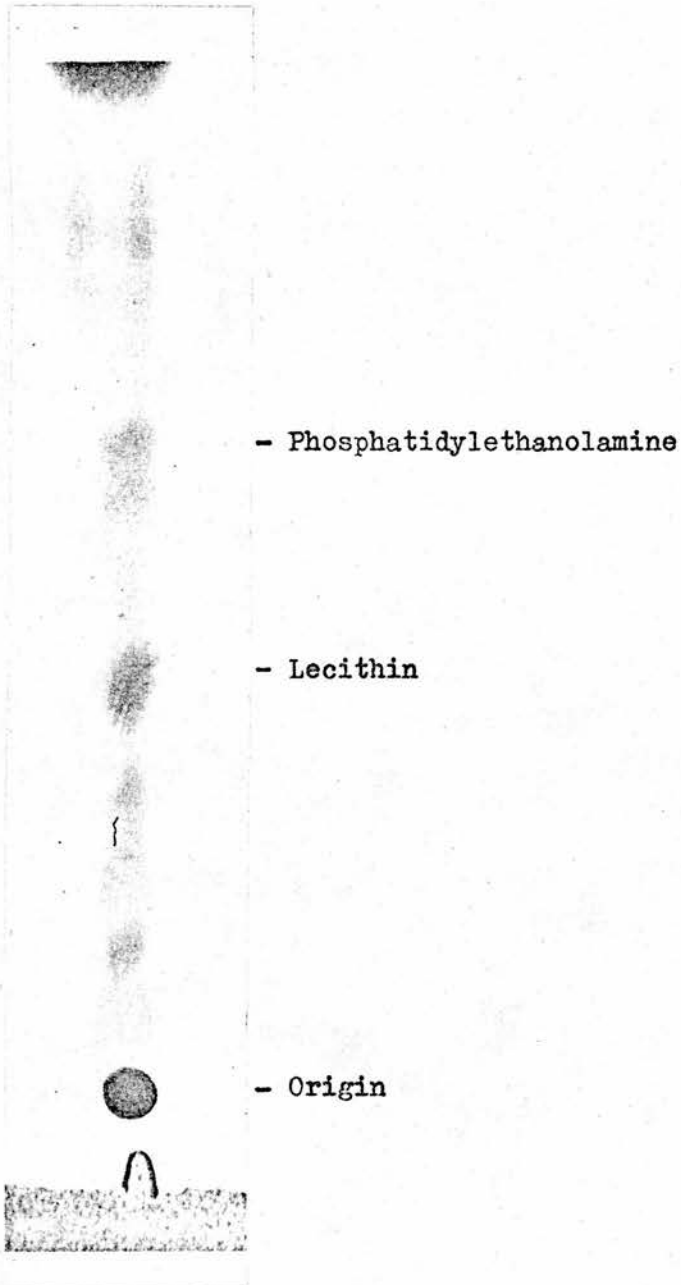


Fig. 42 Thin-layer chromatogram of the phospholipid components of the adrenodoxin proteolipid

A glass plate coated with silica-gel G was used. The solvent system was chloroform:methanol:water::65:25:4.

by centrifugation. The resultant protein preparation which contained cytochrome P450 was lyophilised.

The lyophilised protein was suspended in distilled water and sonicated in the cold (MSE Ultrasonic, Mark 5, 10 x 30 s). A suspension of (impure) lecithin and (impure) phosphatidylethanolamine in distilled water was prepared as described earlier. The protein suspension and phospholipid suspension were each dialysed for 8 hours in the cold against 1,000 vol.distilled water (with one change). The protein extract had little cytochrome P420 present.

The cytochrome P450 was chemically reduced with dithionite and flushed with carbon monoxide before use. Two series of preparations were set up (Table X). No organic solvent was added in these preparations. The preparations were agitated as before on a reciprocal shaker but for longer periods of time. The preparations were shaken first for 15 h in the cold (4°C) and the spectra of the isooctane phases read against a blank of isooctane. Fig. 43 (dotted lines) shows that both preparations showed marked extinction at around 400 nm and 275 nm with increased extinction being shown by the preparation with added phospholipid. The inset in Fig. 43 shows that reading the sample with added phospholipid against the blank (without added phospholipid) shows no marked extinction at 420 nm or 450 nm (dotted line). However, after addition of a little solid dithionite to both sample and blank and passing carbon monoxide through the sample a small but distinct extinction maximum developed at 420 nm (inset - solid line). A further one ml of phospholipid emulsion was added to the sample and both the sample and blank agitated for another 12 hours at 4°C. All the previous extinction spectra increased (Fig. 43, solid lines) with that of the sample with added phospholipid increasing faster than the blank. However the ability to form the spectrum indicative of the

Table X Composition of medium for preparation of proteolipid of cytochrome P_{450}

	Blank	Sample
Protein suspension (cytochrome P_{450}-7.5 μM), ml	6.5	6.5
Phospholipid suspension (390 μg phosphorus/ml), ml	0	0.5
Distilled water, ml	0.5	0
Isooctane, ml	3.5	3.5

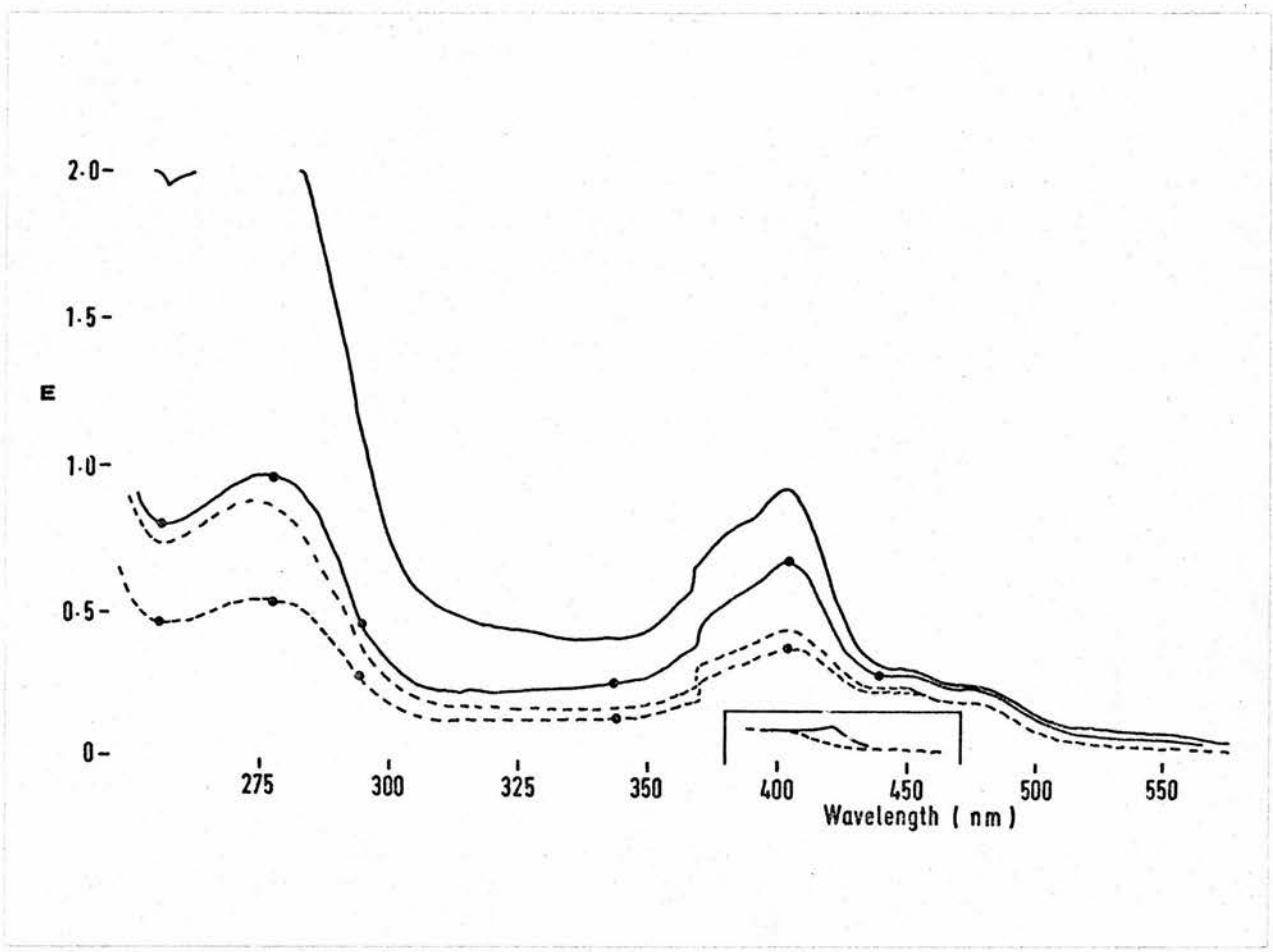


Fig. 43 Spectral characteristics of isooctane solution of proteolipid prepared with cytochrome P450

——————— 1.5 ml phospholipid suspension added. Total shaking time of 27 h

●——————● No phospholipid added. Total shaking time of 27 h

---------- 0.5 ml phospholipid suspension added and shaken for 15 h

●--------● No phospholipid added and only 15 h shaking

The spectra of the above solutions in isooctane were examined against a reference of isooctane.

Inset - The difference spectrum after 15 h shaking of the sample with added phospholipid minus that prepared without added phospholipid is shown by the dashed line. On addition of dithionite to both specimens and carbon monoxide to that with added phospholipid an extinction maximum develops at 420 nm (solid line).

reduced cytochrome P450-CO complex had been lost by this time.

Investigations on protein and phospholipid content of the cytochrome P450 proteolipid

One ml of the isooctane phase of the "sample" (with added phospholipid) and one ml of the "blank" were taken to dryness and the residue redissolved in a dilute solution of sodium cholate and deoxycholate. Biuret determinations of the protein contents were performed using the dried residue from one ml isooctane as reference.

One ml each of "sample" and "blank" were now dried down and redissolved in minimal chloroform:methanol. These specimens were transferred quantitatively onto silica-gel G-coated chromatography plates which were developed with chloroform:methanol:water::65:25:4. In each case much of the phospholipid appeared around the position of phosphatidylethanolamine and lecithin (Fig. 44). (Dr. J. S. Chahl is thanked for a gift of the standard lipids.) The "spots" were scraped off the TLC plates and their phosphorus content determined according to Rouser, Siakotos and Fleischer (1966) (see "Methods"). The results of protein and phospholipid determinations are summarised in Table XI.

The figures shown in Table XI of the analysis demonstrate that whereas exogenous phospholipid serves to increase the amount of proteolipid extracted into the isooctane phase, the complex formed with endogenous and (or) exogenous phospholipid appears to be remarkably constant in containing equal amounts of phosphatidylethanolamine and lecithin and about 26 µg phosphorus of each per mg protein.

Summary

1. Evidence is presented of isooctane-soluble phospholipid complexes with adrenodoxin and crude cytochrome P450.

2. The complex formed with adrenodoxin contains about 4 residues phospholipid per molecule protein. This finding is discussed.
3. The proteolipid formed from cytochrome P_{450} gives rise to a small but distinct extinction at 420 mm on reaction with dithionite and carbon monoxide in isooctane solution. The complex contains equivalent amounts of phosphatidylethanolamine and lecithin equal to about 26 µg phosphorus of each per mg protein extracted into the isooctane phase.

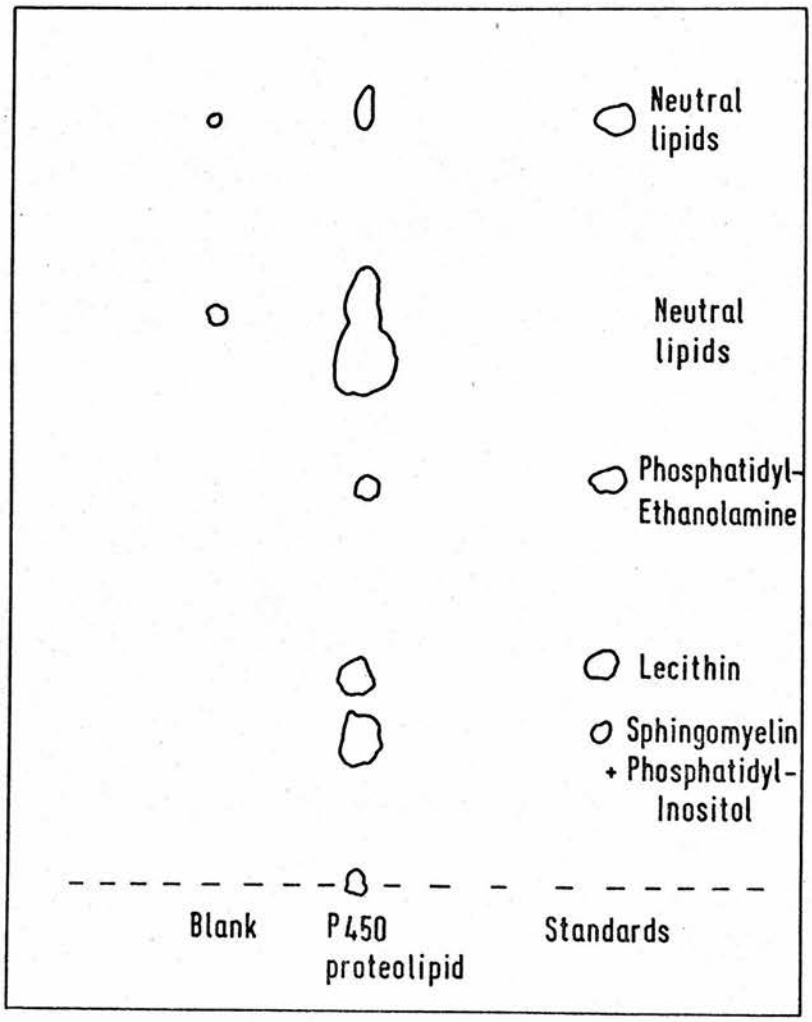


Fig. 44 Thin-layer chromatogram of the phospholipid components of the cytochrome P450 proteolipid

A glass plate coated with silica-gel G was used. The solvent system was chloroform:methanol:water::65:25:4. The "blank" in this Figure related to the isooctane phase of a mixture of phospholipid suspension and isooctane agitated without the addition of a protein component. Under these conditions phospholipids were not extracted into the isooctane phase.

Table XI Composition of proteolipid prepared from cytochrome P450

	Blank (prepared without added phospholipid)	Sample (prepared with added phospholipid)
Phosphatidylethanolamine (μg phosphorus)	103	175
Lecithin (μg phosphorus)	103	175
Protein, mg	3.85	7.0
Phosphatidylethanolamine phosphorus per mg protein, μg	26.8	25
Lecithin phosphorus per mg protein, μg	26.8	25

CHAPTER 8
RESULTS AND EXPERIMENTAL

SOME EFFECTS OF INORGANIC SALTS AND DIPOLAR IONS

In this chapter are considered observations made during investigations into the effects of inorganic salts and dipolar ions. Both these types of compounds influence the dielectric constant of an aqueous solution.

A. Gross effects

The effect of increasing ionic strengths on DOC hydroxylation

Fig. 45 shows the effect of increasing molarities of Tris-HCl pH 7.4 on the 11β-hydroxylation of DOC by a solubilised adrenocortical mitochondrial enzyme extract. It is seen that at pH 7.4 and under the described conditions the activity in 300 mM buffer was only 20 per cent of the maximal activity (which was observed in 100 mM buffer concentration). The 100,000 *g* mitochondrial supernatant used was from an enzyme extract made in 100 mM-Tris-HCl which was dialysed until the ionic strength reached equilibrium at 10 mM buffer.

The reversal by triglycine of the inhibition caused by salt

Fig. 46 shows the 11β-hydroxylation of DOC in the presence of 300 mM-Tris-HCl pH 7.4 and increasing concentrations of triglycine. The other conditions of the incubation were as for the experiment described in Fig. 45. It is seen that the rate of hydroxylation increased linearly with the addition of triglycine up to 50 mM and then reached a plateau. The rate of 11β-hydroxylation in 300 mM-Tris-HCl plus 50 mM-triglycine was double that in the Tris-HCl without the dipolar ion.

Reversal by some dipolar ions of the inhibition caused by salt

Investigation was made as to the effect of adding 50 mM concentrations of various dipolar ions on the inhibition of 11β-hydroxylation of DOC caused by increasing the Tris-HCl content of the medium from 0.1 M to 0.2 M. It is seen that addition of each of these compounds resulted in partial reversal of the inhibition (Table XII).

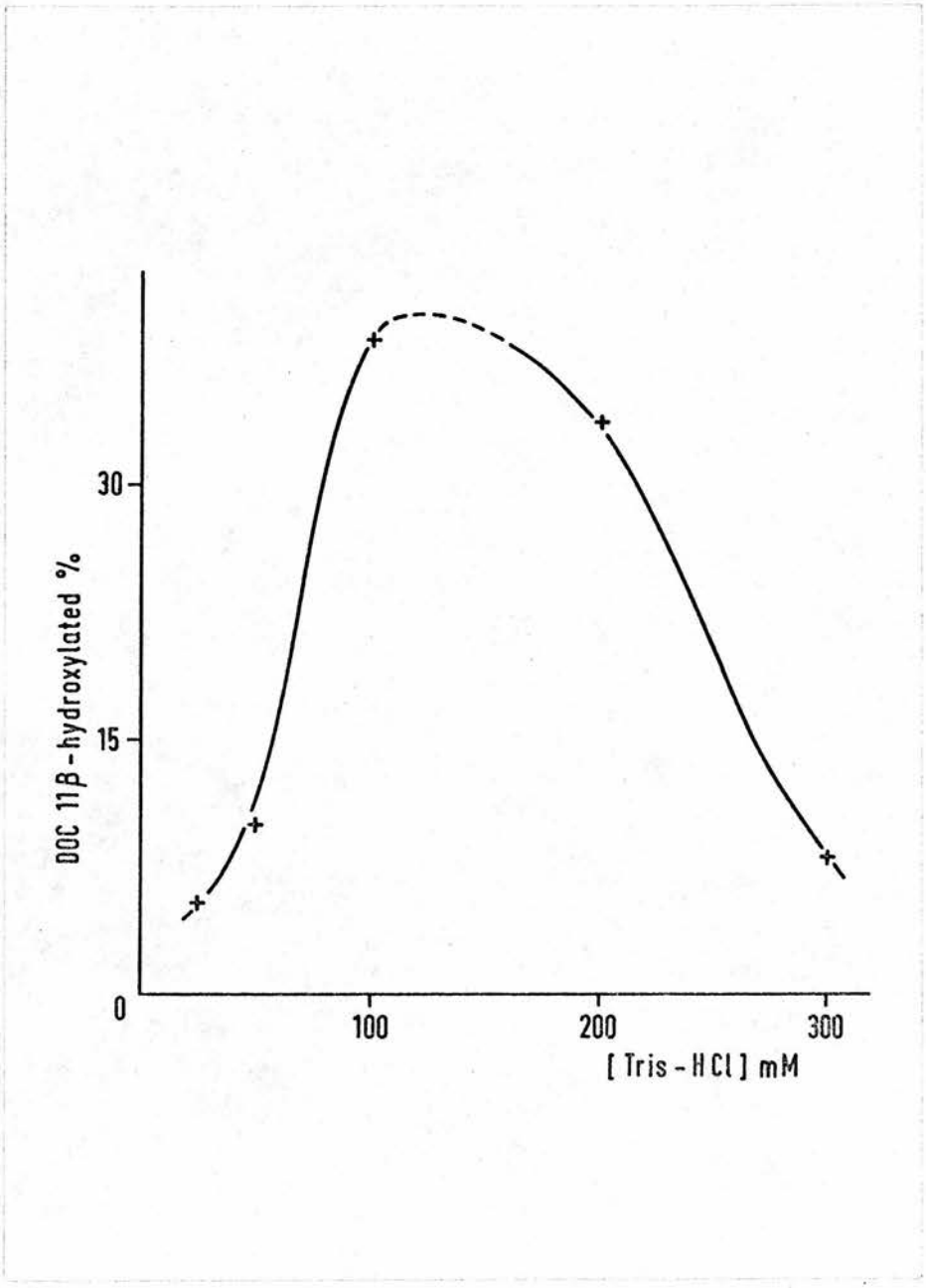


Fig. 45 The variation of rate of DOC 11 β-hydroxylation at different ionic strengths(pH 7.4)

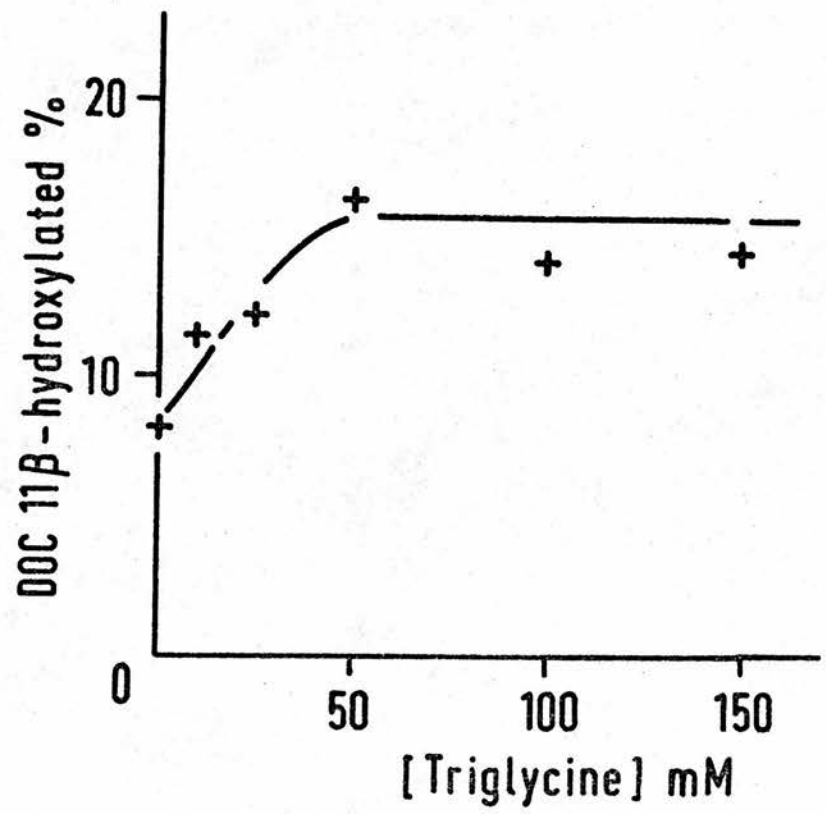


Fig. 46 The effect of triglycine on DOC 11β-hydroxylations inhibited by 300 mM-Tris-HCl

Table XII Effect of some dipoles in reversing the inhibition by salt of DOC 11β-hydroxylating activity

Dipolar ion (50 mM)	Reversal of inhibition %
Glycyl-DL-phenylalanine	1.0
N-acetyl histidine	6.2
ε-amino caproic acid	19.9

Effect of adding triglycine on hydroxylations at various salt concentrations

It was of interest to investigate whether the effects of triglycine described above were applicable over a range of salt concentrations. Fig. 47 shows that addition of triglycine (25 mM) resulted in increased 11 β-hydroxylation at all salt concentrations studied.

Effect of different concentrations of (a) bovine serum albumin, (b) diglycine, and (c) an amino acid on hydroxylations

Since proteins, peptides and amino acids are capable of increasing the dielectric constant of an aqueous solution (Wyman, 1936) the effects of increasing concentrations of bovine serum albumin or diglycine on 11 β-hydroxylation were investigated (Figs. 48 and 49). The similarity of results obtained with either of these agents suggests that similar mechanisms may be operating in each case. Initially at low concentrations of the agent a reduced rate of hydroxylation is observed with augmented hydroxylation at higher concentrations. Fig. 50 shows that an amino acid, histidine, has a similar effect on the rate of side-chain cleavage of cholesterol.

Effects of salts on NADPH-cytochrome c reduction

The ability of adrenal mitochondrial cytochrome P450 reductase (i.e. adrenodoxin plus adrenodoxin reductase) to mediate the transfer of reducing equivalents from NADPH to cytochrome c is utilised as an assay of the activity of the enzyme preparation (see Introduction). The nonenzymic reduction of cytochrome c by NADPH was shown to be negligible. However the addition of ~~80 μg~~ (80 μg protein) cytochrome P450 reductase resulted in rapid reduction of cytochrome c (see also Omura et al, 1966).

Fig. 51 shows the effect of increasing Tris-HCl concentrations on the NADPH-cytochrome c reduction activity of mitochondrial

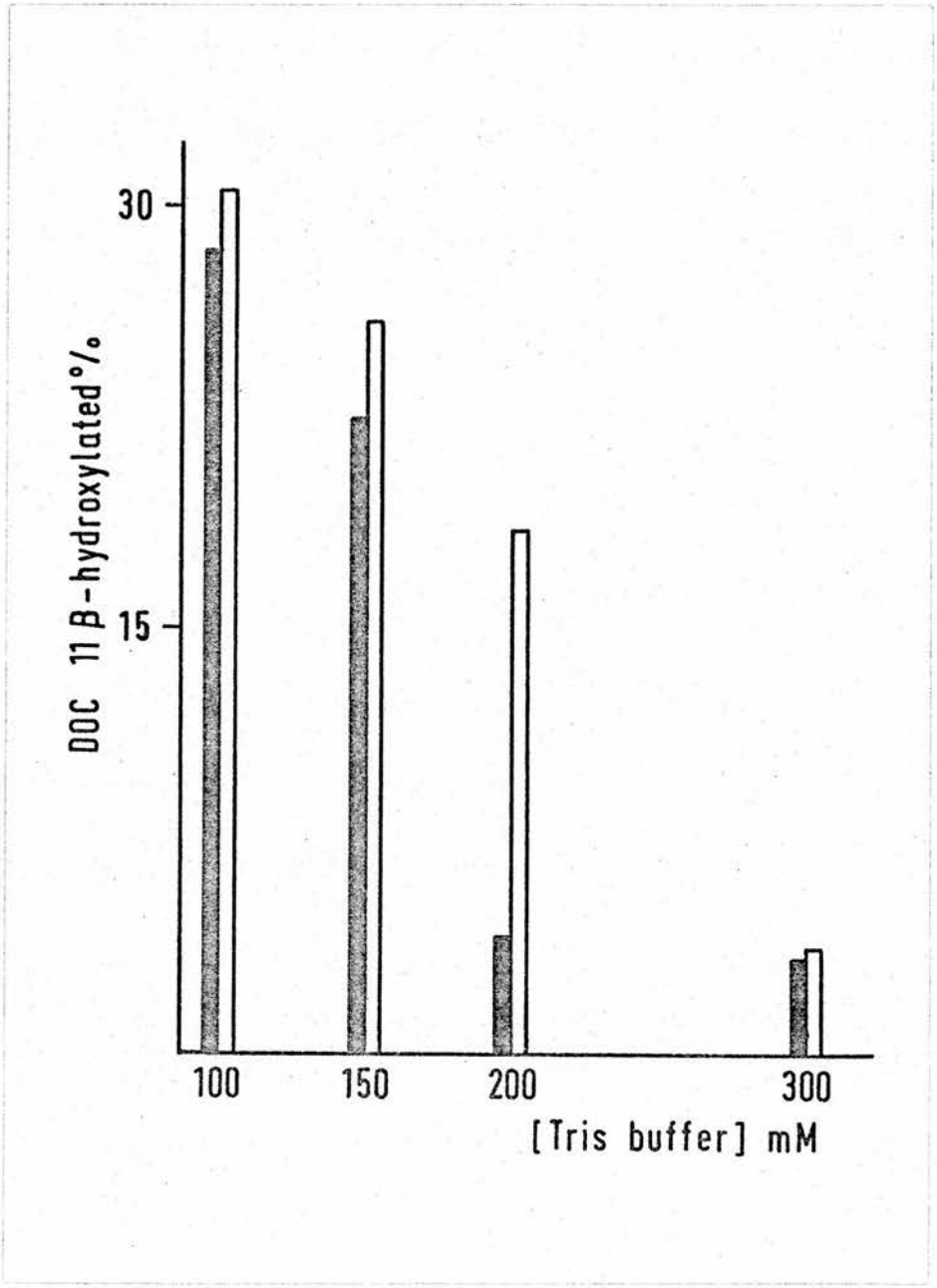


Fig. 47 The effect of triglycine on DOC 11 β-hydroxylations at different salt concentrations

Solid bars - No triglycine added
Open bars - plus triglycine (25 mM)

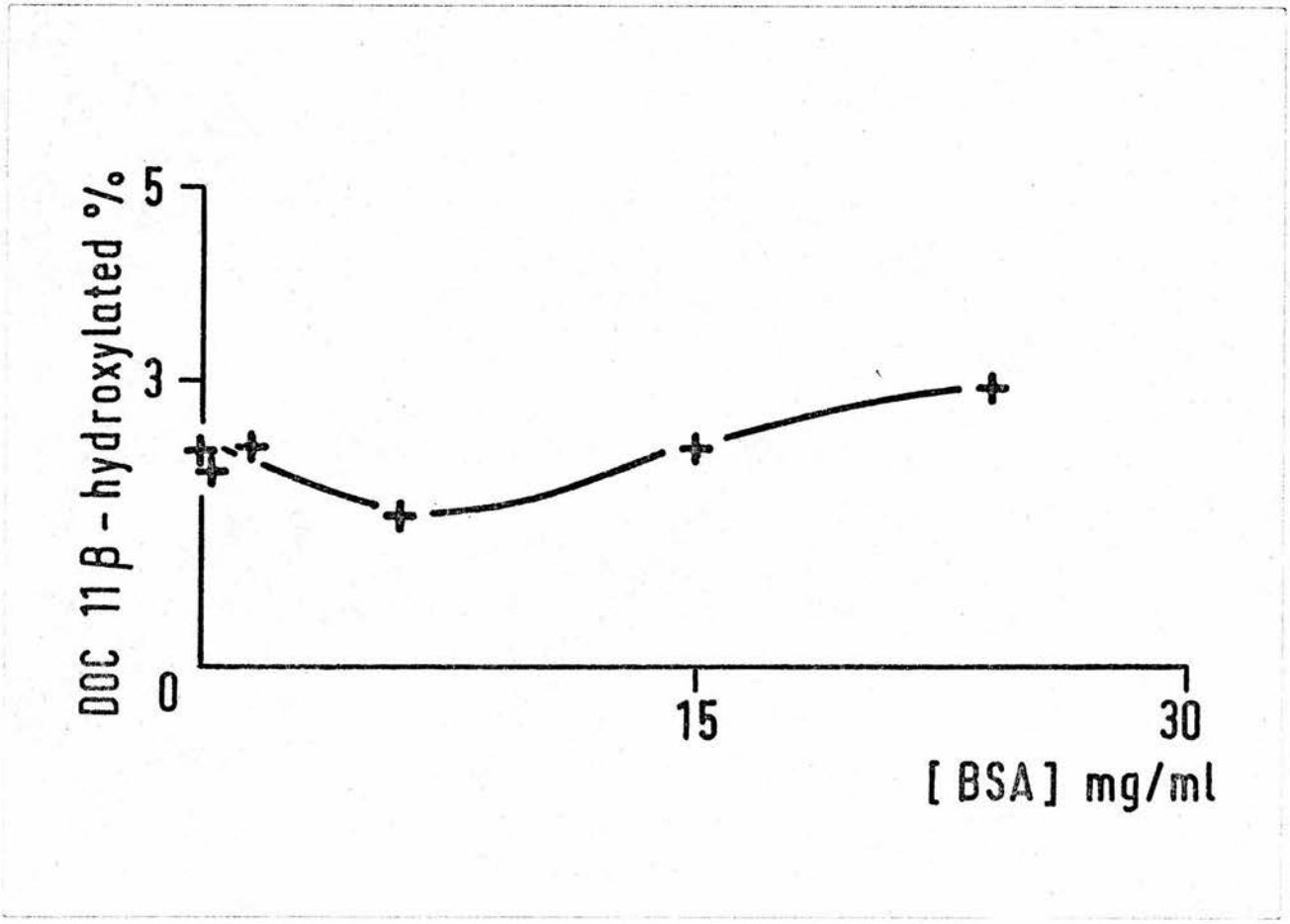


Fig. 48 The activity of DOC 11 β-hydroxylase in the presence of different concentrations of bovine serum albumin at 100 mM-Tris-HCl

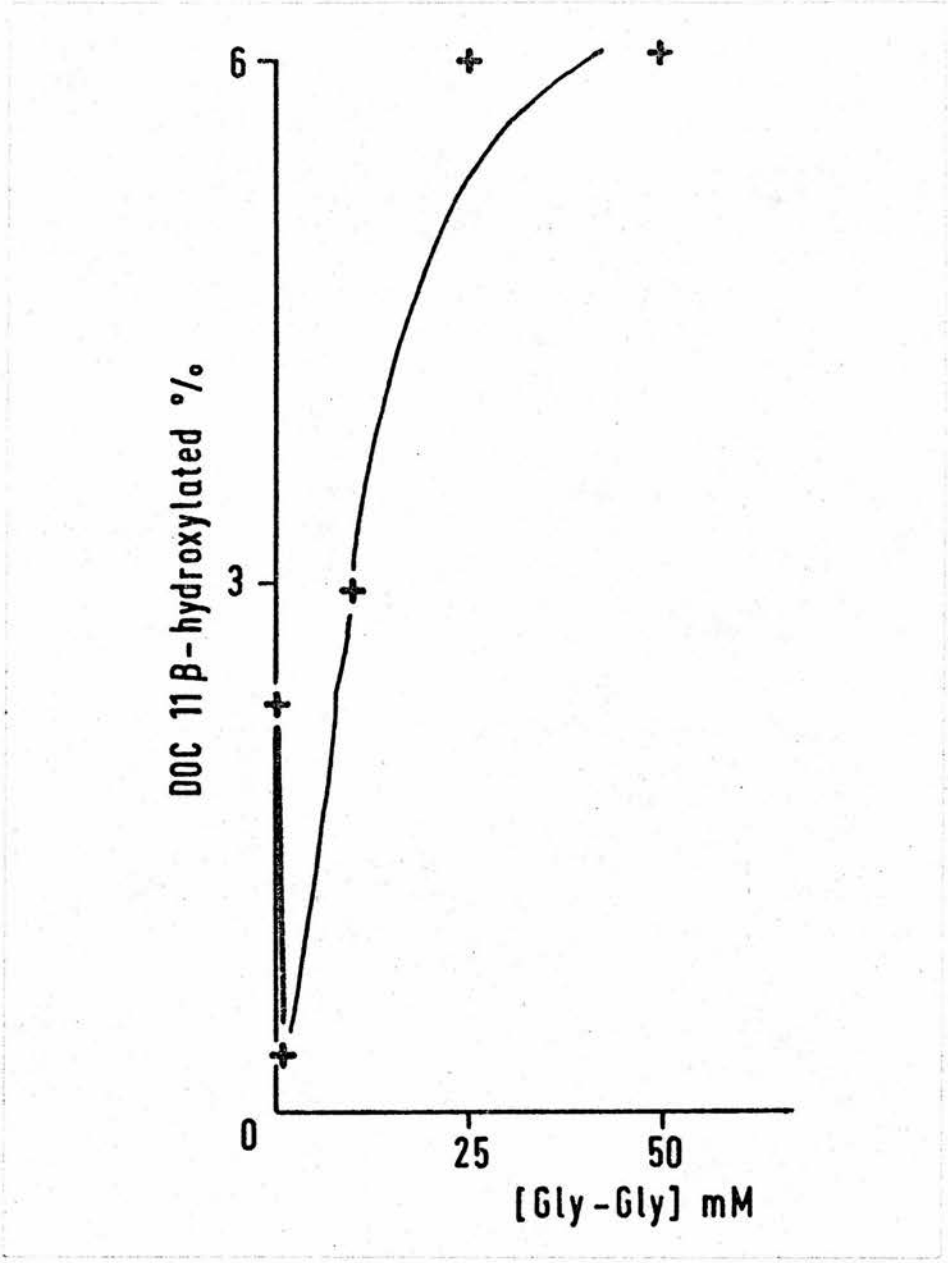


Fig. 49 The DOC 11β-hydroxylating activity at different concentrations of diglycine at 100 mM-Tris-HCl

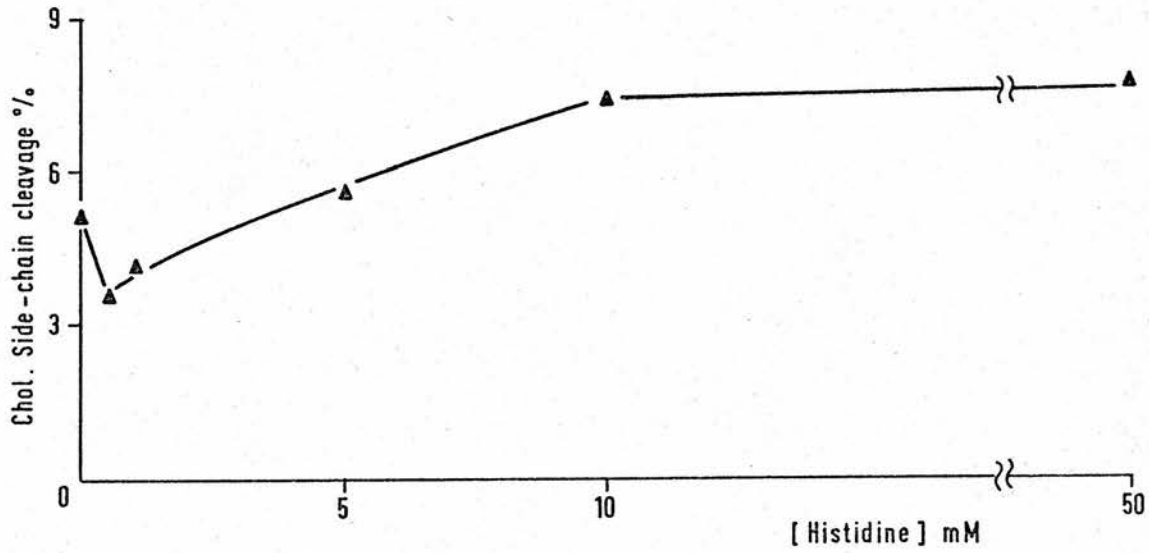


Fig. 50 Cholesterol side-chain cleavage activity at different concentrations of histidine at 100 mM-Tris-HCl

cytochrome P450 reductase with other assay conditions unchanging. The effects are closely similar to the described effects of increasing concentrations of Tris-HCl on 11β-hydroxylation of DOC (Fig. 45). Maximal activity was observed around 50 mM-Tris-HCl and that at 300 mM was about 14 per cent of the maximal.

Fig. 52 shows that a chloride of a divalent cation has a more marked effect than that of a monovalent cation. Magnesium chloride inhibited cytochrome c reduction more markedly than chlorides of monovalent cations: of these the effect of potassium chloride is shown in Fig. 52. These assays were carried out in 100 mM-Tris-HCl. Other studies also showed that the sulphate ion had a more marked inhibitory effect than the chloride ion.

It was found that the reduction of DCPIP by NADPH in the presence of adrenodoxin reductase also appeared to be affected (though to a lesser extent) by salt concentrations. The significance of this was difficult to assess since salts are reported to affect hydrogen ion transfer to DCPIP (Webb, 1963, p.835). Similar reactions mediated by ferredoxin reductase are also however known to be sensitive to raised ionic strengths as is the complex formed by this protein with ferredoxin (Nelson and Neumann, 1969b).

Effect of salt on the association of adrenodoxin and its reductase

From the preceding data it appeared possible that the effect of ionic strength on steroid hydroxylations was mediated at the level of the transfer of reducing equivalents rather than at the locus of oxygen-insertion into the steroid molecule. It has been shown (Chapter 5) that the association of adrenodoxin and the flavoprotein does not appear to result in the formation of a stable compound of fixed stoichiometry as observed on titrating one of the proteins into a solution of the other. The effect of ionic strength on the association of a fixed amount of each protein was now investigated.

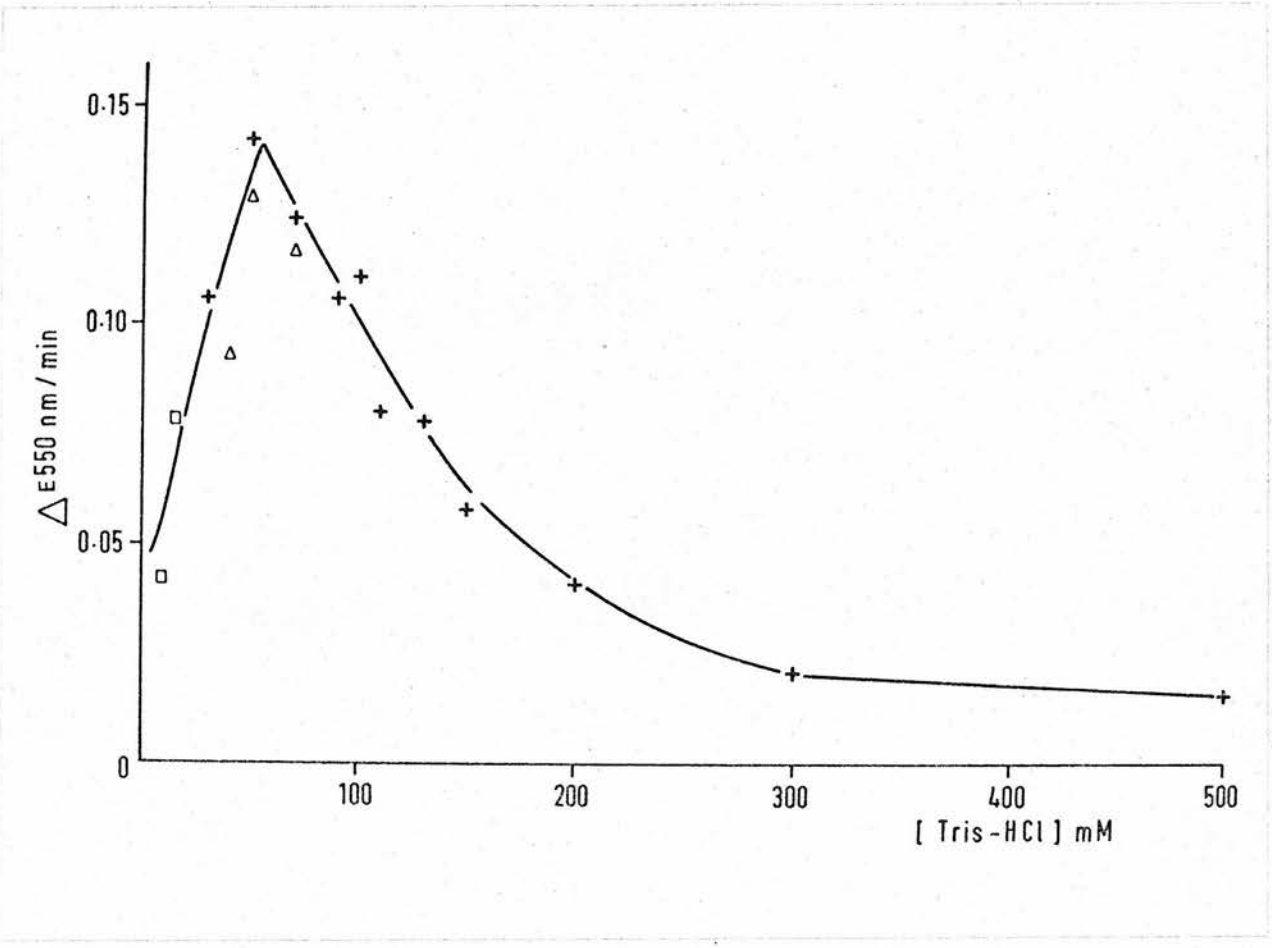


Fig. 51 The variation in rate of cytochrome c reduction by adrenodoxin reductase plus adrenodoxin at different ionic strengths

The different symbols denote different preparations of Tris-HCl buffer, pH 7.4.

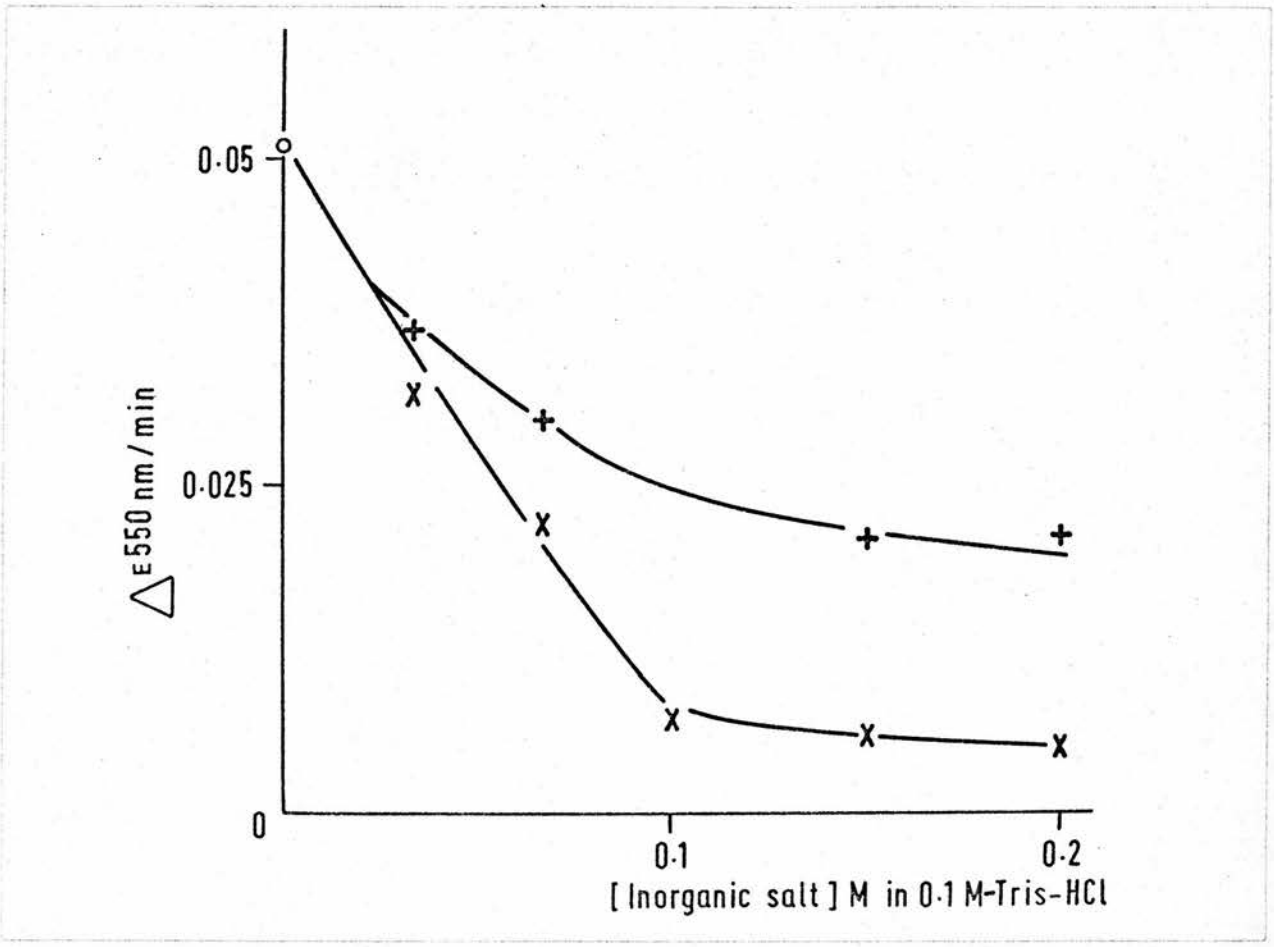


Fig. 52 The effect of different concentrations of potassium chloride and magnesium chloride on the rate of cytochrome c reduction by adrenodoxin reductase plus adrenodoxin

+——+ rates of reduction in the presence of potassium chloride

X——X rates of reduction in the presence of magnesium chloride

The assays were performed in 0.1 M-Tris-HCl, pH 7.4. The rates of cytochrome c reduction in the presence of sodium chloride or ammonium chloride were comparable to those in the presence of potassium chloride.

Two silica cells were lined up in the "reference" light path and another two in the "sample" light path of a Unicam SP 800 spectrophotometer. 2.5 ml of purified flavoprotein (2.3 mg protein/ml) in 10 mM-Tris-HCl pH 7.4 was placed in one cell in the "reference" light path and in one cell in the "sample" light path. 2.5 ml adrenodoxin ($E^{415nm}:E^{280nm}$ = 0.8 , 0.28 mg protein/ml)in 10 mM-Tris-HCl pH 7.4 was added to each of the remaining cells. The contents of the two cells in the "sample" light path were mixed and redivided equally between the two cells. The spectral extinction of the mixed proteins against their component solutions was scanned from 700 to 320 nm. A spectrum was obtained with gradually increasing extinction at wavelengths below 500 nm and a small minimum at about 400 nm.

Equal aliquots of 1 M-sodium chloride were added to each of the four cells, the contents of which were then well stirred. The contents of the two cells in the "sample" path were mixed and redivided equally into the same cells.

The change in extinction at 400 nm following addition of increasing amounts of sodium chloride is shown in Fig. 53. Each reading was corrected for dilution and each point represents the change in extinction at 400 nm caused by addition of sodium chloride to the association of adrenodoxin and flavoprotein minus any effect of sodium chloride on the separate proteins. It is to be noted that a plateau was reached at about 60-70 mM-NaCl under the described conditions.

Effects of NaCl and KCl on the interaction of adrenodoxin and cytochrome P450

Fig. 54 shows the results from similar experiments using 2.2 ml "solubilised" cytochrome P450 (5.5 mg protein/ml) and 2.2 ml adrenodoxin ($E^{415nm}:E^{280nm}$ = 0.8, 0.18 mg protein/ml). Equimolar sodium chloride and potassium chloride have different effects on the changes in inter-

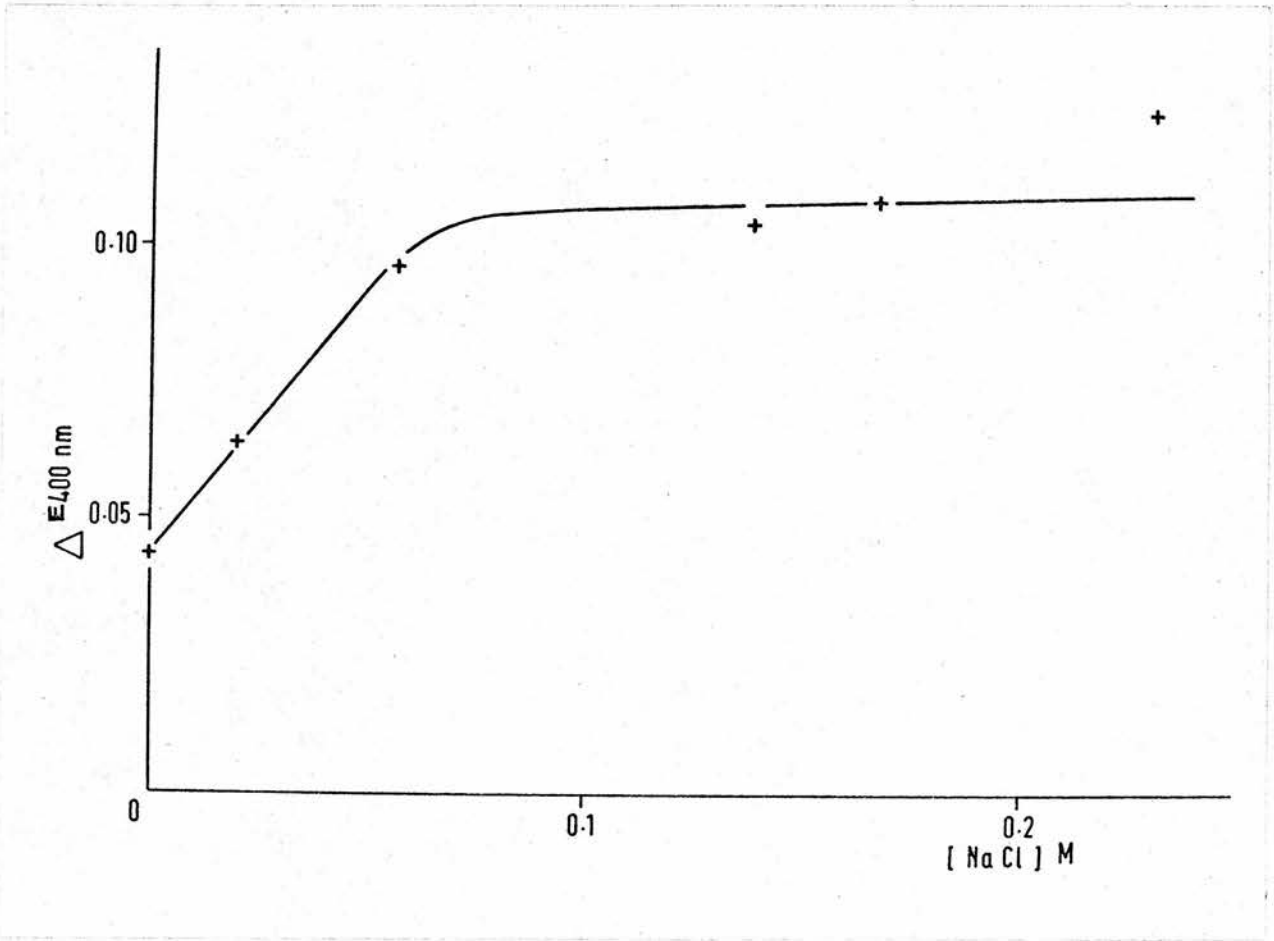


Fig. 53 The effect of increasing concentrations of sodium chloride on the light extinction at 400 nm of a mixture of adrenodoxin reductase and adrenodoxin at 10 mM-Tris-HCl, pH 7.4

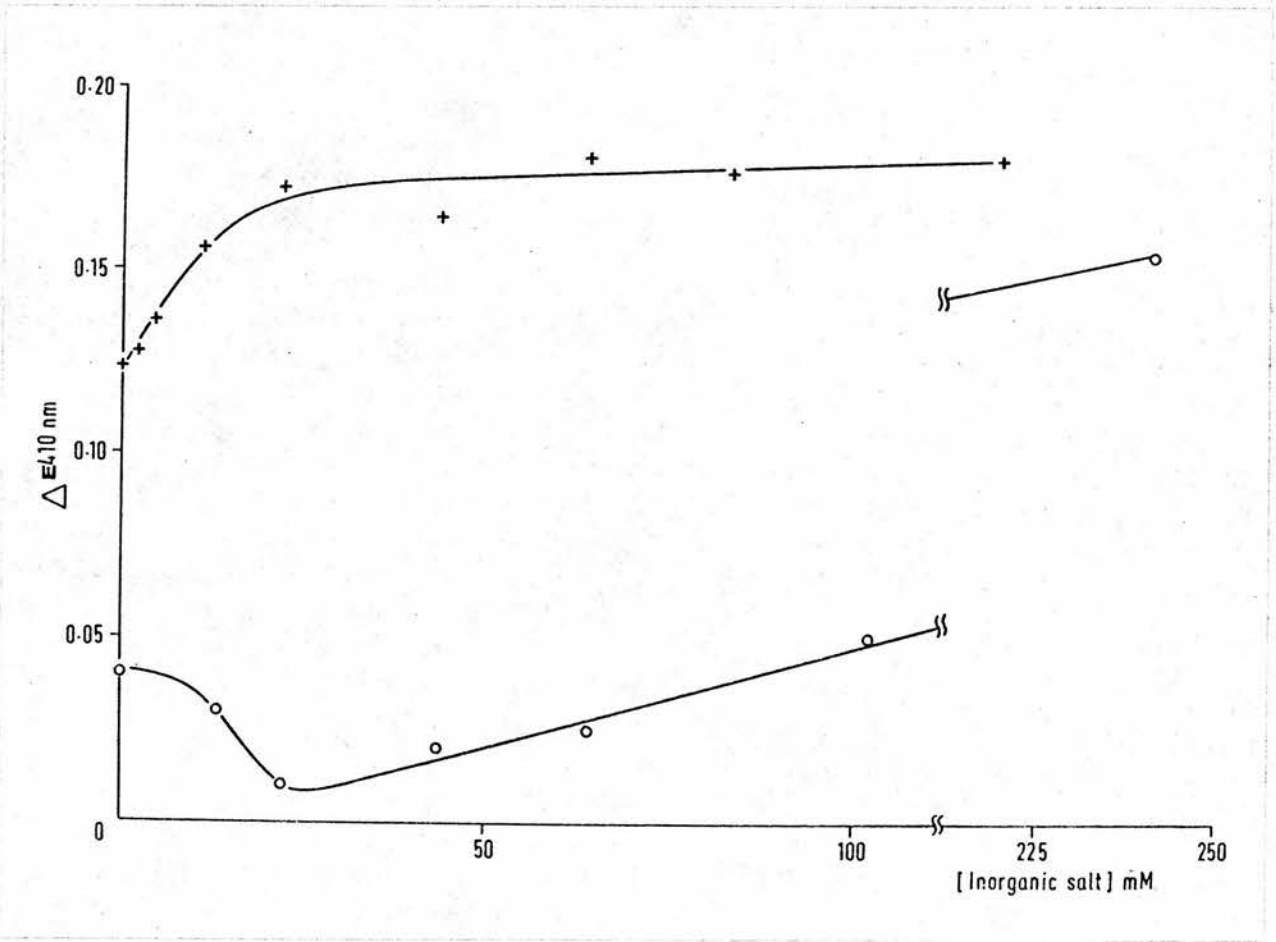


Fig. 54 The changes in light extinction at 410 nm of mixtures of adrenodoxin and cytochrome P450 due to increasing concentrations of sodium chloride or potassium chloride at 10 mM-Tris-HCl, pH 7.4

\+ ——— + Effects of the addition of sodium chloride

0 ——— 0 Effects of the addition of potassium chloride

action when other conditions are kept constant. The readings were corrected for dilution.

B. Fine effects

A relationship of the extent of inhibition by various ions

It was of interest to examine whether various inorganic monovalent ions in high concentrations were capable of inhibiting the mitochondrial steroid hydroxylations. It had been shown earlier that maximal 11β-hydroxylation of DOC was obtained at an ionic strength of 100 mM-Tris buffer and that higher concentrations inhibited the reaction (Fig. 45). Investigation was therefore made of the effect of adding 0.15 M concentrations of various salts to enzyme assays in 0.1 M-Tris buffer.

The enzyme preparation in 0.1 M-Tris-HCl pH 7.4 was dialysed overnight against 0.1 M-Tris-HCl and sonicated briefly (MSE Mark 5, 2 x 1 min) before use. All assay reagents and salt solutions were also made up in 0.1 M-Tris buffer pH 7.4 and the pH of each reagent solution and the reaction mixtures checked.

The effect of solutions of LiCl, NaCl, KCl, NH_4Cl and CsCl were examined in assays of 11β-hydroxylation of DOC at 37.5°C for 15 min. Examination was also made of the effects of adding various halides of sodium in incubations at 37.5°C for 10 min.

Addition of equimolar amounts of different salts was found to result in the inhibition of the maximal rate of hydroxylation to different extents. If the relative inhibitions caused by the various chlorides were plotted as a function of the ionic radii of the respective cations a straight line was found to result. Similarly a plot of the inhibitory effects of the various halides of sodium as a function of the ionic radii of the respective halide ions also gives a straight line tending in the same direction as the previous line (i.e. the sign of the charge on the ion does not appear to be of much

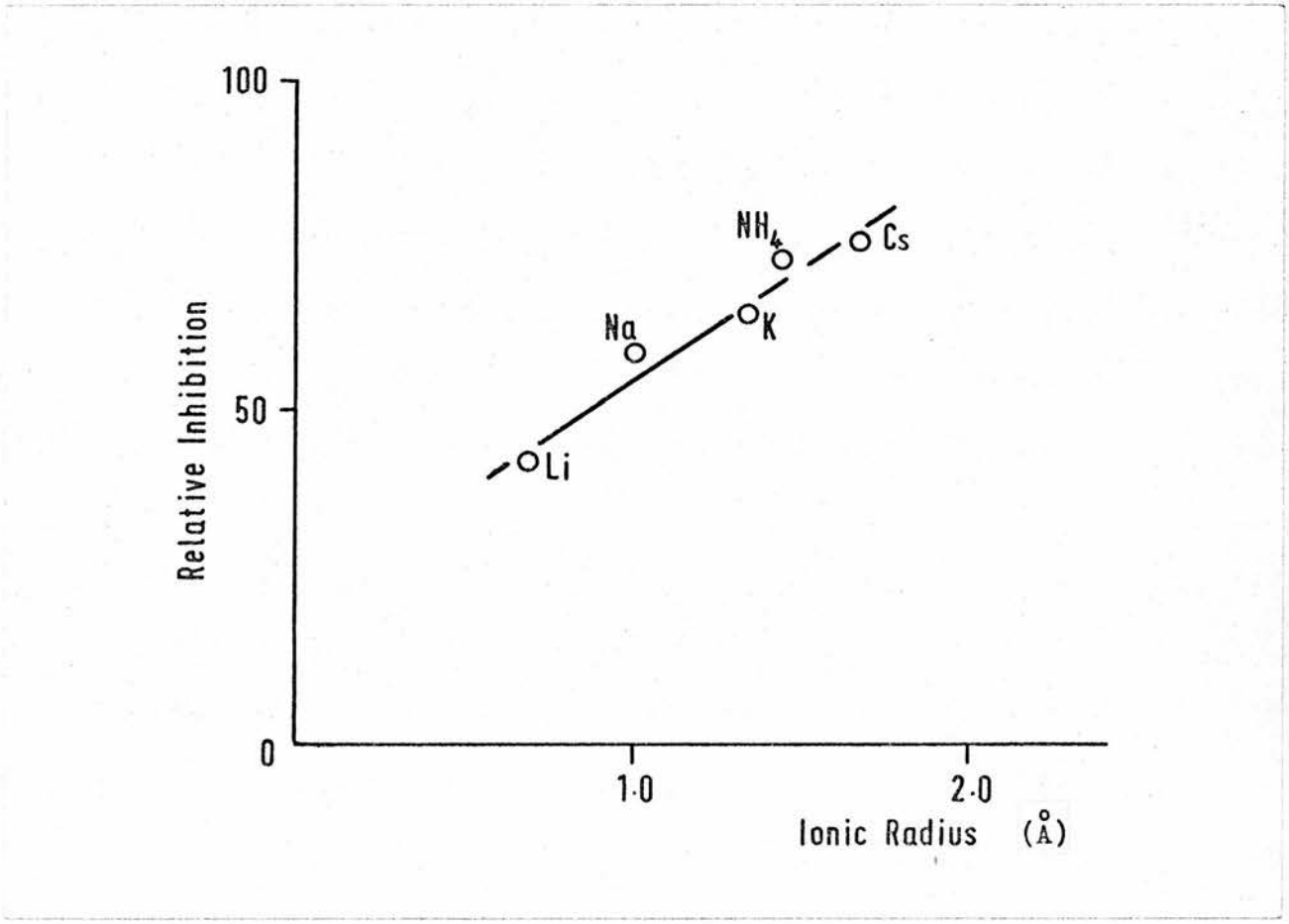


Fig. 55 The relationship between the relative inhibitions of DOC 11 β-hydroxylation due to salts of monovalent ions and the radius of the cations

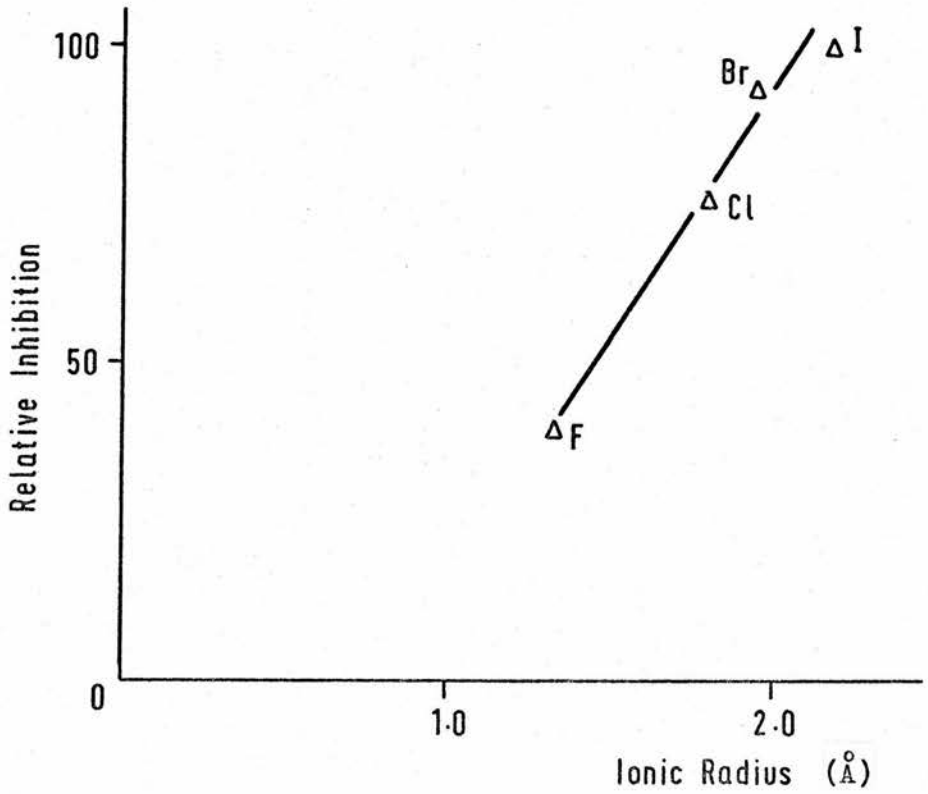


Fig. 56 The relationship between the relative inhibitions of DOC 11 β-hydroxylation due to salts of monovalent ions and the radius of the anions

significance). Figs. 55 and 56 demonstrate the kind of relationships observed with the different monovalent cations and anions respectively.

Effect of different periods of preincubation of enzyme with salt

The effects of various salts described above may be due to an effect of ionic strength on the course of the enzymatic reaction or to an inactivation of the proteins of the enzyme system. If the latter explanation is correct preincubation of the enzyme system with salt may progressively result in increased inhibition. Investigation was therefore made of the effect of preincubating the enzyme in incubation mixtures containing 0.1 M-NaCl in 0.1 M-Tris buffer for a given time before starting the reaction with the addition of the NADPH generator. Preincubating the enzyme in this manner for 0, 2, 5, 21 and 46 min did not however cause significant differences in the rate of DOC 11β-hydroxylation determined subsequently.

Effect of duration of incubation on relative inhibition observed

The relative inhibition referred to above was calculated by the following formula:-

$$\text{Relative inhibition by ionic strength} = \frac{\text{DOC hydroxylation in 0.1 M-Tris} - (\text{DOC hydroxylation in 0.1 M-Tris} + x\text{M-salt})}{\text{DOC hydroxylation in 0.1 M-Tris}} \times 100$$

However the inhibition is not referred to as the percentage inhibition as the absolute values are influenced by the conditions under which a given series of incubations were made. Fig. 57 shows the time courses of DOC 11β-hydroxylation in 0.1 M-Tris-HCl and in 0.1 M-Tris-HCl plus 0.1 M-NaCl. Fig. 58 shows a plot of data derived from the previous Figure whereby it is seen that addition of 0.1 M-NaCl produced a 65 per cent inhibition in an incubation of 2 min duration but only about 25-30 per cent inhibition in one of 90 min. The explanation of

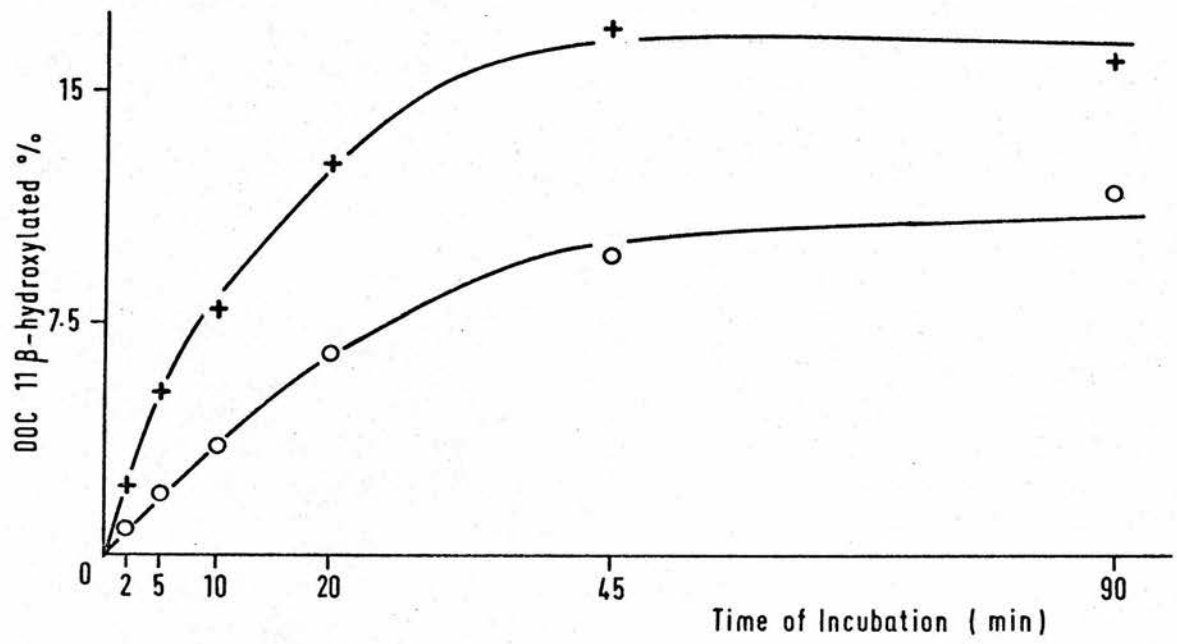


Fig. 57 Time course of DOC 11β-hydroxylation activity at different ionic strengths

+ ——— + Incubations performed in 0.1 M-Tris-HCl, pH 7.4

0 ——— 0 Incubations performed in 0.1 M-Tris-HCl, pH 7.4 plus 0.1 M-NaCl

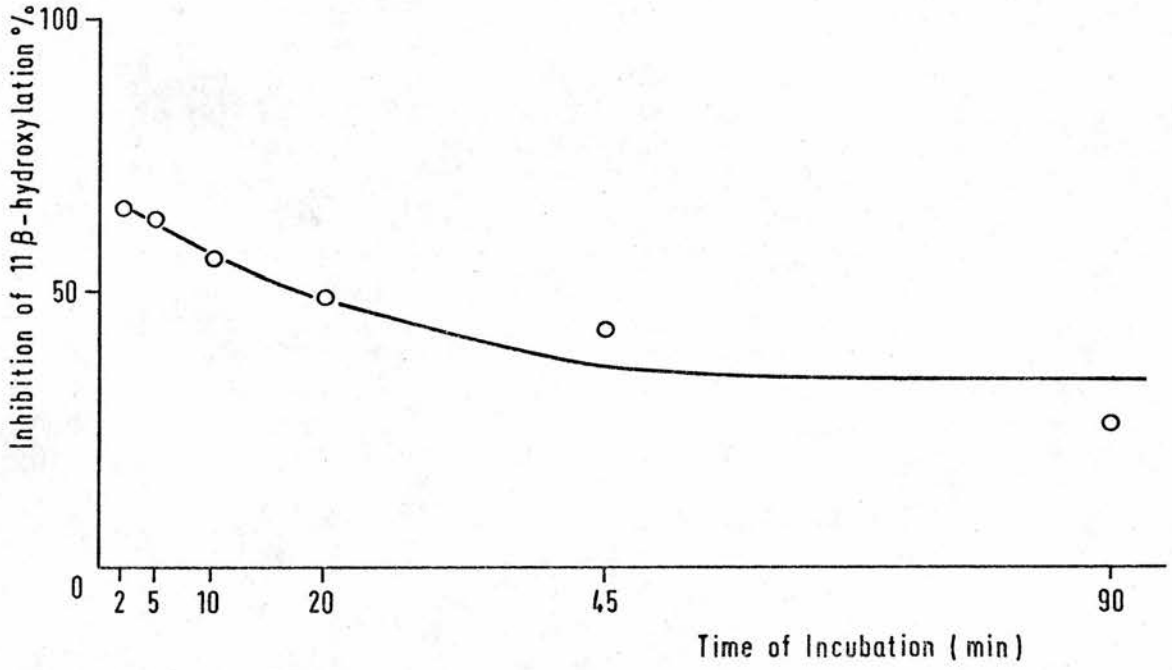


Fig. 58 The effect of the duration of incubation on the percentage inhibition by ionic strength of DOC 11 β-hydroxylation

this observation is not apparent at this stage but needs to be borne in mind in evaluating results obtained on different series of incubations.

Summary

1. The maximal DOC 11β-hydroxylating activity of adrenal mitochondrial enzyme was achieved in about 100 mM-Tris-HCl with inhibition at higher ionic strengths.

2. Several dissimilar dipolar ions partially reversed the inhibition caused by different ionic strengths.

3. Bovine serum albumin, peptides and amino acids inhibit the 11β-hydroxylation of DOC and cholesterol side-chain cleavage in low concentrations and activate them at higher concentrations.

4. The ability of adrenal mitochondrial cytochrome P450 reductase to effect NADPH-cytochrome c reduction is affected by ionic strength in a manner similar to the effect of ionic strength on DOC 11β-hydroxylation. Inhibition of cytochrome c reduction by divalent ions is more marked than that by monovalent ions.

5. The association of adrenodoxin with the flavoprotein and cytochrome P450 was found to be affected by inorganic salts. The effects of equimolar NaCl and KCl were not the same.

6. The extent of effect of salts of monovalent ions on 11β-hydroxylation of DOC appears to be related to the ionic radius of the component ions.

CHAPTER 9

DISCUSSION

The objectives of this investigation were to examine the components of adrenal mitochondrial cytochrome P450 reductase and investigate some of the factors which could affect the supply of reducing equivalents at the steroid hydroxylating active centre.

The experimental results present a technique developed for the preparation of highly purified adrenodoxin reductase as judged by Sephadex gel-filtration and disc-gel electrophoresis. The percentage amino acid composition of the flavoprotein is presented. It does not show the abundance of acidic residues which are a feature of adrenodoxin (Table VI) and cytochrome P450 (Gunsalus et al., 1969). The nitrogen content of the flavoprotein and a comparison of results obtained by different methods of protein estimation are also presented. Miniature electrofocussing of the protein showed the isoelectric point of the principal DCPIP-reducing activity to be near pH 8.9. The pH for maximal DCPIP-reducing activity of the enzyme is about 6.5. The behaviour on electrofocussing confirms the absence of a high proportion of acidic residues in the protein and explains why unlike adrenodoxin the flavoprotein is not tightly bound to DEAE-cellulose at pH 7.4 (Fig. 26; Omura et al., 1966).

The DEAE-cellulose ion-exchange chromatography and ammonium sulphate fractionation under buffered conditions at pH 7.4 employed in the purification of adrenodoxin reductase was found to result in loss of ability to catalyse the reduction of DCPIP by NADPH. Although treatments specifically designed to liberate the prosthetic group had not been employed it was found that the NADPH-DCPIP reductase activity was immediately regained on adding FAD to the inactive enzyme preparation. The maximal reactivation of an inactive preparation was found on addition of 1-10 μM-FAD to a standard DCPIP-reductase assay containing 0.19 mg adrenodoxin reductase (Fig. 22).

Higher concentrations of FAD resulted in progressive inhibition of enzymatic activity. In previous studies, the activity of histidine transaminase from Escherichia coli B was shown to be increased by addition of the cofactor, pyridoxal phosphate, which also appeared to cause inhibition when used in excess (Wickramasinghe, Hedegaard and Roche, 1967; Wickramasinghe, 1969). The inhibition by excess FAD in the present studies could be due to excess flavin (a) non-competitively inhibiting the enzyme, (b) blocking the binding-sites for NADPH or DCPIP by binding onto the enzyme, (c) interacting nonenzymatically (Yagi, Ozawa and Okada, 1959) with the substrate DCPIP or (d) diverting the flow of reducing equivalents by acting as a substrate which could then undergo autoxidation. Competition for binding sites could not however be demonstrated between FAD and DCPIP using the assay for DCPIP-reducing activity. Neither could bleaching of free flavin be demonstrated on substituting substrate quantities of FAD for DCPIP in this (aerobic) assay and observing the extinction at 450 nm over several hours. The reduction of free flavin was however satisfactorily demonstrated under strictly anaerobic conditions (Fig. 23). A 1.18 to 1.0 stoichiometry of NADPH added to FAD reduced proved that the flavin was indeed being reduced at the expense of the reduced pyridine nucleotide and partial reoxidation of the FAD was observed on momentary exposure to the air of the deaerated reaction mixture. The effect of FAD on steroid hydroxylations by enzyme preparations derived from adrenal mitochondria was also examined. The 100,000 g supernatants from solubilised acetone powder (for cholesterol side-chain cleavage incubations) or lyophilised mitochondria (for 11β-hydroxylation of DOC) were used and in each case whereas a small increase in hydroxylating activity may have occurred at the lower concentrations of FAD the most striking effect was the inhibition of

hydroxylation at FAD concentrations higher than about 15 nmol FAD per mg protein (Figs. 24 and 25). In view of the earlier results on the effect of excess FAD on the DCPIP-reducing activity of the flavoprotein it may be postulated that the inhibitory effect of the free flavin on steroid hydroxylation is by acting as a "bleed" of reducing equivalents from the flavoprotein with the FAD being rapidly reoxidised by dissolved oxygen. It was noticed that the spectrophotometric assay system for measuring the DCPIP-reducing activity of the flavoprotein required concentrations of over 160 nmol FAD per mg protein for inhibition while inhibition of steroid hydroxylation took place from over 15 nmol FAD per mg protein. An explanation for this could be that the hydroxylation assay is carried out under conditions of vigorous aeration which results in rapid reoxidation of reduced FAD. Furthermore the utilisation of reducing equivalents for steroid hydroxylations is slower than that for DCPIP-reduction. Although the above findings are interesting in themselves and in the study of the flavoprotein, adrenodoxin reductase, it is not however suggested on the basis of the observations described that the reduction and reoxidation of free FAD in high or low concentrations has any physiological significance in the functioning of steroid hydroxylation in adrenal cortex mitochondria.

~~The next enzyme in the mitochondrial hydroxylating chain is~~ adrenodoxin and considerable effort was spent in purifying and investigating some of the properties of this protein. One of the earliest characteristics to be investigated was its molecular weight. Filtration on a carefully calibrated Sephadex G200 column showed it to be excluded at the same position as was cytochrome c which would be consistent with ~~an assessment of~~ molecular weight of around 12,400. This ~~figure was at variance with the figure of 15-20,000 (Kimura and~~

The next enzyme in the mitochondrial hydroxylating chain is adrenodoxin and considerable effort was spent in purifying and investigating some of the properties of this protein.It has been seen (page 77) that preparations obtained in the course of this work had $E^{415nm}:E^{280nm}$ ratios of 0.81.However Kimura's methods of preparation has regularly yielded protein of $E^{415nm}:E^{280nm}$ of about 0.76 and Omura et al.(1967) obtain about 0.8, while Orme-Johnson and Beinert (1969c) have reported preparations with $E^{415nm}:E^{280nm}$ ratio as high as 0.88.These results conflict with molar extinction coefficients at 414 nm and 276 nm published by Kimura (1968) which give a maximal $E^{414nm}:E^{276nm}$ ratio of 0.754.

One of the earliest characteristics of the purified protein to be investigated was its molecular weight.Filtration on a carefully calibrated Sephadex G200 column showed it to be excluded at the same position as was cytochrome c which would be consistent with a molecular weight of around 12,400.This figure was at variance with the figure of 15 - 20,000 (Kimura and

(continued on next page-

Suzuki, 1967) which was commonly accepted at the time this study was undertaken. The figure of 15-20,000 was in itself a reduction of earlier estimates of 22,000 but being however higher than the molecular weights of 11-12,000 reported for many ferredoxins (see Introduction) the present independent verification was undertaken. The value obtained and reported here was confirmed in subsequent publications by Kimura (1968) and Kimura et al. (1969) by iron analysis to be 13,600; by amino acid composition to be 11,000; three different sedimentation equilibrium techniques to be 12,000 and sedimentation diffusion to be 11,300. The average of these values amply confirms the finding here reported that adrenodoxin, like many ferredoxins, has a molecular weight of around 12,000.

The electrofocussing of adrenodoxin resulted in two areas of denatured protein coming out of solution at about pH 5.0. A comparable effect was noted by Koch et al. (1970) when Soya bean nodule bacteroid ISP came out of solution at pH 6.0 during studies on its isoelectric point. These observations are explained by the high content of acidic amino acid residues in these proteins (see TableIII) which cause them to migrate under the influence of an applied voltage to the acid regions of a pH gradient. Many other proteins are also known to be less soluble and come out of solution at their isoelectric point. Adrenodoxin and other ISP^{s} may have the same tendency or it may be that the acid conditions destroyed the iron-sulphur chromophores and denatured the proteins. Koch et al. (1970) in fact found that electrofocussing of the bacteroid ISP resulted in a complete loss of enzymatic activity which was only marginally restored on treatment with sodium sulphide, ferrous ammonium sulphate and mercaptoethanol (Malkin and Rabinowitz, 1966a). It had been hoped that the miniature electrofocussing technique would be of use in reaching a decision as to the existence of isoenzymes of adrenodoxin but the insolubility of

adrenodoxin at or near its isoelectric point limited the usefulness of this method.

Since the involvement of adrenodoxin is known in cholesterol side-chain cleavage and in 11β- and 18-hydroxylations (Nakamura, Otsuka and Tamaoki, 1966; Omura et al., 1966) in adrenal mitochondria and since there is evidence of the existence of more than one species of cytochrome P450 it is interesting to speculate as to whether physically very similar but functionally distinct isoenzymes of all three proteins are present. Since isoenzymes of some ferredoxins (e.g. in Leucena glauca) have been identified through study of their amino acid sequences (see Introduction) it was considered possible that more than one form of adrenodoxin may exist with their principal variations being in biological function.

Four indications initially suggested that more than one form of adrenodoxin exists. (a) Omura et al. (1965a) reported that most of the adrenodoxin tended to precipitate at 35-55% ammonium sulphate saturation although more still remained in the supernatant. Kimura and Suzuki (1967) found that adrenodoxin was precipitated between 60-80% saturation while the present studies confirmed that 85% or more ammonium sulphate saturation is needed for complete precipitation. While individual techniques and conditions of fractionation do vary it is unusual for a single protein to show the wide range of precipitation from 35-85% ammonium sulphate saturation. This suggests the possibility of protein heterogeneity. However the finding during the course of this work that increasing salt concentrations affect the binding of adrenodoxin to its reductase (Chapter 8) provides an explanation as to why during ammonium sulphate fractionation of crude protein extracts some of the adrenodoxin is brought down with its reductase at relatively low salt saturation. (b) The high amount of adrenodoxin

(as estimated by its light extinction at 415 nm) required to reconstitute the steroid hydroxylating chain could be partly due to the presence of more than one species of this protein. The other two observations were (c) the absence of any published electrophoretic data of "purified" adrenodoxin preparations despite the considerable amount of other findings reported on this protein, and (d) the finding that during ion-exchange chromatography some adrenodoxin appears to be desorbed at somewhat lower ionic strength than the rest (although two distinct peaks could not be separated). Since both the electrophoretic and ion-exchange behaviour of a protein depend principally on its ionisation properties, it was decided to employ these techniques to try to separate different species of adrenodoxin and examine their biological activity. Some of the approaches used to resolve this question were described in Chapter 4. No conclusive evidence however was obtained by these means. More positive indications were however noted on comparison of the amino acid composition of adrenodoxin as prepared in this study with those prepared by Kimura's group (Kimura, 1968; Kimura et al, 1969) and Yasunobu's group (Tanaka, Haniu and Yasunobu, 1970). Although the amino acid composition of the three preparations is very similar in most respects (Table VI), especially in view of the larger number of residues per molecule allowed for in Yasunobu's calculation / based on an abnormally high molecular weight the content of aspartic and glutamic acid residues varies considerably between the three preparations. The differences indeed are not solely quantitative (i.e. as regards numbers of residues of aspartic and glutamic acids per molecule) but in the present preparation the glutamic content is significantly higher than that of aspartic acid while in Kimura's and Yasunobu's figures the aspartic residues outnumber the glutamic residues. Since it is well established that adrenal cortex microsomal steroid hydroxylations are not dependent on

adrenodoxin and are indeed inhibited by it (Sweat et al., 1969) and no evidence has been found of the presence of an ISP in the adrenocortical extramitochondrial fractions (Masters et al., 1971; Sweat et al., 1969), it is unlikely that these discrepancies in the amino acid composition of bovine adrenodoxin are the result of contamination by an extramitochondrial ISP. It therefore is possible that this finding provides an indication of the possible presence of more than one type of adrenodoxin in the bovine adrenal cortex. It is yet to be seen whether differences in amino acid composition of different adrenodoxin preparations can be correlated with different steroid hydroxylating processes since adrenodoxin is in all cases only concerned with the one enzymatic function of the transfer of reducing equivalents from adrenodoxin reductase to cytochrome P450. Again the differences observed between ferredoxins isolated from different individuals of the same species of tree, Leucena glauca, and similar studies on other plant ferredoxins (see Introduction) indicate that variations in structure may exist between ISP molecules with the same biological function. Differences in amino acid composition of adrenodoxin may be related to interaction with adrenodoxin reductase and cytochrome P450 of different specificities.

The interaction of components of various enzymatic electron-transferring systems and reconstitution of these systems have been described in several cases. The chief interest in several laboratories has perhaps been focussed on the components and reconstitution of systems relating to mitochondrial electron transport (e.g. Fowler and Richardson, 1963; Green, 1961; Singer and Gutman, 1970), succinic dehydrogenase (e.g. Wang and Wang, 1964) and photosynthetic electron transport pathways (e.g. Nelson and Neumann, 1969b). Green (1961) gives a detailed account of the separation of four "complexes"

of the classical liver mitochondrial electron transport chain and their recombination in relatively simple proportions to reconstitute all or part of the activities of the chain. The initial report (Cooper, Schleyer and Rosenthal, 1968) on a similar type of recombination of components of the electron transfer system concerned with adrenocortical mitochondrial steroid hydroxylation showed however that maximal activity was obtained only with a stoichiometry of about 50 parts of adrenodoxin to each one of cytochrome P450 and flavoprotein. Mitani and Horie (1970) also found the requirement of a high proportion of adrenodoxin for maximal activity in in vitro mitochondrial steroid hydroxylase reconstitution experiments. Cooper, Schleyer and Rosenthal (1968) drew attention to the possibility that the low redox potential of cytochrome P450 may contribute to this unexpected stoichiometry.

The redox potentials of the mitochondrial steroid hydroxylating system and closely related enzymes are of considerable interest in that they are apparently very low and resemble each other. Whereas hepatic microsomal cytochrome P420 was reported by Omura and Sato (1964b) to have a redox potential of -20 mV, Waterman and Mason (1970) found a value of -410 mV for hepatic microsomal cytochrome P450. Adrenodoxin has been variously reported to have a redox potential of +164 mV (Kimura and Suzuki, 1967), -370 mV (Hall and Evans, 1969) and at least 400 mV less than +164 mV (Suzuki and Estabrook, 1968 quoted in Orme-Johnson and Beinert, 1969c). It is now generally accepted that adrenodoxin has a very low redox potential as is the case with spinach ferredoxin (-420 mV) and clostridial ferredoxin (-390 mV) (Tagawa and Arnon, 1968; see also Introduction). The redox potential of adrenodoxin reductase is yet to be investigated but Erecinska et al.(1970) find those of pigeon-heart flavoproteins(on the substrate side of the rotenone-block) to range from -220 to -260 mV. Wilson and Dutton's

(1970a,b) recent investigations demonstrate that other electron-transferring cytochromes have much higher redox potentials than cytochrome P450. This low redox potential (i.e. about -400 mV) of the terminal oxygen-accepting cytochrome P450 compared to the redox potential of about -320 mV of the physiological donor of reducing equivalents, NADP(H) (Burton, 1957) is surprising. The redox potentials of these proteins may differ somewhat from the values indicated above. However the highly negative nature of the potentials of these three enzymes is generally accepted. It is therefore possible that when these three enzymes are separated from their intramitochondrial associations (with each other and the mitochondrial membrane) and "solubilised", their unusual relationship of redox potentials do contribute to the high proportions of adrenodoxin required to obtain maximal steroid hydroxylation in an in vitro assay system. In concluding discussion of this possibility it should also be noted that the redox potentials of the isolated proteins under laboratory conditions may not be those which exist in the physiological state. Indeed Sobel and Lovenberg (1966) found a systematic variation of redox potential of ferredoxin with pH and suggested this may have a regulatory function, with which however Tagawa and Arnon (1968) do not agree.

Another possible influence on the stoichiometry of "reconstitution" appears to be indicated by the experimental data presented in Chapter 5. It was demonstrated spectrophotometrically that adrenodoxin interacts with the adrenodoxin reductase protein of the hydroxylating chain and there is considerable difference in the amount of flavoprotein which needed to be added to obtain similar spectra in the case of titrations carried out in distilled water and those carried out in 0.1 M-phosphate buffer. A preliminary report has also been made of the interaction of

adrenodoxin with spinach ferredoxin reductase (see Foust, Mayhew and Massey, 1969). The interaction of plant ferredoxins with ferredoxin reductases have been reported to take place at 1:1 and 2:1 ratios by Foust and Massey (1967) and Nelson and Neumann (1969b) respectively with formation of complexes with characteristic difference spectra. The difference in reported stoichiometry has been suggested (Nelson and Neumann, 1969b) as being due to the former experiments being performed in 30 mM-phosphate and the latter in 5 mM-Tris buffers. This complex was dissociated progressively on addition of salts, which also inhibit enzymatic reactions in which the complex participates (Foust, Mayhew and Massey, 1969; Nelson and Neumann, 1969b). Investigations were therefore made of the correlation of reconstitution stoichiometry with steroid hydroxylating activity achieved in different incubation mixtures as regards ionic strength and a protein which is not a component of steroid hydroxylases (bovine serum albumin). It was found that the composition of the assay medium had a considerable effect on the activity of the reconstituted systems (Figs. 31 and 32) and therefore must be taken into account in attempting to assess reconstitution of these multiprotein systems by measuring rates of substrate conversion by the preparation. Experimental evidence is also presented (Fig. 35) to show that exogenous adrenodoxin added to a 100,000 g mitochondrial supernatant augments its ability to effect steroid hydroxylation (whether cholesterol side-chain cleavage or DOC 11β-hydroxylation). This is significant since much of the cytochrome P450 and flavoprotein extracted from adrenal mitochondria is found in the 100,000 g pellet while the (soluble) adrenodoxin remains in the supernatant. It would therefore seem from these results that all the adrenodoxin from a given quantity of adrenal cortex mitochondria is inadequate to saturate the cytochrome P450 and flavoprotein remaining

in a 100,000 g supernatant under the conditions of an *in vitro* assay of steroid hydroxylation.

Protein-protein interaction is also known to occur in other unrelated systems and the Michaelis-Menten reaction scheme type approach has been successfully applied to the study of those complexes. Pronounced effects of ionic strength and (or) bovine serum albumin on other reconstitution experiments and (or) the stoichiometry of the complexes concerned have been reported for the reconstitution of the mitochondrial electron transfer system (Fowler and Richardson, 1963), pepsin-serum albumin complex (Cann and Klapper, 1961), cytochrome c-cytochrome c peroxidase (Nicholls and Mochan, 1971) and the 1:1 complex of cytochrome c with mitochondrial structural protein (Edwards and Criddle, 1966) in addition to those on the complex between ferredoxin and ferredoxin reductase (Foust and Massey, 1967; Foust, Mayhew and Massey, 1969; Nelson and Neumann, 1969b). Nichol *et al.*(1964) in discussing the question of interacting protein systems make the point that in the interaction of proteins higher order complexes may be formed depending on the conditions of the reaction.

The preceding discussion and much of the results obtained in the present investigation point to the rate at which reducing equivalents reach the locus of hydroxylation on cytochrome P450 as being a key mechanism in the regulation of corticosteroidogenesis in adrenal mitochondria. Peron and McCarthy (1968) review some of the evidence suggesting in fact that an action of ACTH is in controlling the supply of electrons. Investigation was therefore made of some factors which may affect electron donation. Fig. 5 includes some of the metabolic systems which could affect this electron supply.

It was confirmed that cholesterol side-chain cleavage and the 11β-hydroxylation of DOC by sonicated mitochondrial enzyme was

supported by NADPH as electron donor. Inhibition of 11β-hydroxylation of DOC was marked at NADPH concentrations above the optimal level of 0.15 mM. Satoh, Constantopoulos and Tchen (1966) and Tchen (1968) reported similar inhibition of adrenal cholesterol side-chain cleavage activity with 0.5 mM-NADPH but Mason (1970) did not find this with placental cholesterol side-chain cleavage activity. Tchen's (1968) observations showed adrenal cholesterol side-chain cleavage to have maximal activity at an NADPH concentration of 0.05 mM which contrasts with the present observations that the optimal NADPH concentration for 11β-hydroxylation of DOC is 0.15 mM. Another difference between the two NADPH-dependent hydroxylations was that the Km for NADPH for the 11β-hydroxylation of DOC was about 34.5 μM while that for cholesterol side-chain cleavage was about 2 μM. The latter value is close to one of 1 μM reported by Mason and Boyd (1971) as the Km for NADPH for cholesterol side-chain cleavage by placental mitochondrial enzyme compared with an apparent Km for NADPH of 300 μM for hepatic microsomal cholesterol 7α-hydroxylase (Boyd, 1970).

Consideration of the above results leads to some interesting possibilities. The inhibition of steroid hydroxylation by excess NADPH is possibly related to the inhibition by excess NADPH of the rate of cytochrome c reduction mediated by adrenodoxin and adrenodoxin reductase (Chapter 5). The rate of cytochrome c reduction is also inhibited by excess adrenodoxin and in fact a large excess of adrenodoxin appears to lessen steroid hydroxylation in reconstitution experiments (Chapter 5). NADPH and ferredoxin at high concentrations similarly inhibit electron transfer reactions mediated by the plant flavoprotein, ferredoxin-NADP reductase (Nelson and Neumann, 1969a). It is interesting to speculate whether the different sharp optimal concentrations and Km values for NADPH for the two steroid hydroxylations have regulatory implications.

The differences could be construed as suggesting the existence of different flavoproteins for the two reactions as well. More work needs however to be carried out on this problem before attempting to understand the deeper physiological implications since it is generally accepted that numerous factors control or affect the intramitochondrial levels of NADPH (Peron and McCarthy, 1968).

The evidence shown in Chapter 6 shows that the NAD-kinase in the bovine adrenal cortex cell resides largely in the cytoplasm. The level of activity is of the same order as that of the liver cytoplasmic enzymes which is understandable since the oxidation and reduction of NADP(H) in the course of its functions as a cofactor would not be expected to lead to a requirement for its rapid replenishment. Similarly preliminary experiments did not indicate an effect of ACTH of increasing NAD-kinase activity either in loose-cell preparations or in vivo. While NADPH is not considered to readily pass through intact mitochondrial membranes, McKerns (~~in McKerns,~~ 1968 ~~p.479~~) considers that the intramitochondrial reduction of NADP to NADPH by glucose-6-phosphate dehydrogenase is activated by ACTH.

The effect of another physiological reducing agent, reduced glutathione, in slightly increasing 11β-hydroxylation at the lowest concentrations is unlikely to be due to an action as an electron donor. The locus of this action and that of the inhibition by higher concentrations of reduced glutathione and all concentrations used of oxidised glutathione is uncertain. It would not appear to be on adrenodoxin since thiols are known to affect hepatic microsomal steroid hydroxylations (Scholan, 1969) where no adrenodoxin-like protein has been demonstrated. Sulimovici (1968) found similar results using rat ovarian mitochondria but Mason (1970) did not find a marked effect of concentrations up to 10 mM-reduced glutathione on placental

cholesterol side-chain cleavage. Neubert (1966) has described effects of reduced and oxidised glutathione on swelling of mitochondria which may have relevance to hydroxylations in physiological conditions.

The physiological and structural state of the hydroxylase electron transport chain within the mitochondrion may also be related to the finding that at least two of these three proteins form isooctane-soluble phospholipid complexes. The interaction of adrenal mitochondrial cytochrome P450 with phospholipid had previously been postulated by Williamson and O'Donnell (1969) following their finding that adrenal mitochondrial lipid and asolectin enhance steroid hydroxylation and NADPH oxidation by adrenal mitochondrial enzyme preparations. Their interpretation that phospholipid stabilises the haemprotein and is required for the maintenance of its unique spectral and enzymatic properties is based on these findings and those of previous workers (e.g. Omura and Sato, 1964a,b) that treatment designed to remove phospholipid also converts cytochrome P450 to the enzymically inactive cytochrome P420. Evidence published subsequent to completion of the present study show also however (Billiar et al, 1971) that both cholesterol side-chain cleavage and DOC 11β-hydroxylase activities can be released from adrenal cortex mitochondria by treatment with phospholipase A. This finding could suggest that not only cytochrome P450 but adrenodoxin and flavoprotein as well are associated with phospholipid on the mitochondrial membrane since an active steroid hydroxylating enzyme system is found in the supernatant after the "digestion", and may be compared to the release of cytochrome c from the electron-transport particle by phospholipase treatment (Ambe and Crane, 1959). The formation of cytochrome c-phospholipid complexes have been intensively studied (Das and Crane, 1964; Das, Haak and Crane, 1965; Michelazzi, 1955, 1957; Reich and Wainio, 1961).

Edwards and Criddle (1966), Green and Tzagoloff (1966) and Williams and Thorp (1970) suggest that phospholipid bonds form a means by which cytochrome c is (loosely) linked to other mitochondrial proteins. Green (1961, 1966) envisages cytochrome c (and also coenzyme Q) as a mobile coenzyme functioning as a shuttle of electrons from one fixed (enzyme) complex to the next. He makes the point that cytochrome c, coenzyme Q, succinate, reduced NAD and O_2 are the only oxidation-reduction components in the mitochondrial electron-transfer system which are not fixed in position within a complex and observes that it is intercomplex electron flow rather than intracomplex electron flow which is the rate-limiting factor (Green, 1961, 1966).

That the enzymic stability and conformation of many native enzymes is assured by their phospholipid content has been shown by studies on, among others, the flavoprotein, succinic dehydrogenase (Cerletti et al, 1970), the hepatic microsomal cytochrome P450-dependent drug metabolising system (Chaplin and Mannering, 1970), adenyl-cyclase (Birnbaumer et al, 1970), cytidine diphosphocholine transferase (Fiscus and Schneider, 1965) and β-hydroxybutyric dehydrogenase (Sekuzu, Jurtshuk and Green, 1963). It is possible that phospholipids help to maintain the stability and the molecular conformation of the individual enzymes of the adrenal mitochondrial steroid hydroxylating systems. However phospholipids may have another function as well. From the present work and experience reported by others (e.g. Boyd and Simpson, 1968; Omura et al, 1966; Yago and Ichii, 1969) it is apparent that mitochondrial cytochrome P450 is firmly membrane-bound while adrenodoxin is a soluble protein. The flavoprotein can be partially solubilised by sonication but there is considerable NADPH-DCPIP reductase activity in cruder cytochrome P450 preparations and while adrenodoxin may be present in solution in the inner matrix (Yago and Ichii, 1969)

several reports (including those of Ichii, Omata and Kobayashi, 1967; Sweat, Young and Bryson, 1970; Yago and Ichii, 1969) agree that the flavoprotein and cytochrome P450 are closely associated on the mitochondrial membrane. It is therefore possible that the soluble adrenodoxin "transports" reducing equivalents from the flavoprotein to cytochrome P450 in a rate-limiting step very similar to that proposed by Green (1966) for cytochrome c. It has been found in this work that a molecule of adrenodoxin complexed with about 4 atoms of phosphorus in the form of phospholipid while the stoichiometry for phospholipid to cytochrome c has been reported to be about 22-32:1 (Chapter 7). A loose phospholipid bonding could serve to maintain the approximate positioning of adrenodoxin relative to the other two proteins while still permitting freedom of movement to transfer reducing equivalents from flavoprotein to haemprotein. In addition, the demonstration of the ability of the cytochrome P420 proteolipid (which is formed from cytochrome P450 under the conditions described) /to be reduced and form a CO-complex in isooctane solution is of interest since within the intact mitochondrion the function of steroid hydroxylation by cytochrome P450 probably takes place in a lipid environment.

Two theoretically possible means, by which the soluble adrenodoxin functionally links the membrane-bound flavoprotein and cytochrome P450 by the transfer of electrons are shown schematically in Fig. 59. The first, Scheme A, envisages adrenodoxin as being capable of physically interacting with both flavoprotein and cytochrome P450 at the same time. This possibility could necessitate the invoking of the postulated semiconduction in proteins for which several theories have been formulated and studies in fact made on cytochrome c as a protein molecule which may act as the path of the electron movement between the site of reduction and the site of oxidation (see Rosenberg and Postow, 1969).

Scheme B appears to be a more attractive possibility in that it would fit in with many described experimental observations. The finding that the cytochrome P450-phospholipid complex is reducible by dithionite in isooctane solution (Fig. 43) suggests that its physiological reduction by adrenodoxin may also take place in an all-lipid environment as well. The shuttling of adrenodoxin between the flavoprotein and cytochrome P450 in a rate-limiting step would suggest a reason for the presence of this protein in mitochondrial corticosteroid hydroxylations (see Table I) and explain many experimental observations. For instance, the rate-limiting nature of this step in an ordered system of fixed stoichiometry of enzyme components including one loosely-linked adrenodoxin molecule could provide a fundamental reason as to why in an enzymatic assay high proportions of unlinked soluble adrenodoxin with 100,000 g mitochondrial supernatant or purified enzymes results in an increased rate of steroid hydroxylation (see Chapter 5). Increasing the concentration of adrenodoxin in an "un-ordered" system (i.e. with adrenodoxin no longer constrained by phospholipid bonds to a functional unit in a 1:1:1 stoichiometry with flavoprotein and cytochrome P450) is effectively to increase the concentration of "substrate" which can be reduced and oxidised by the flavoprotein and cytochrome P450 respectively. This postulates that adrenodoxin is capable of being attached to, reduced or reoxidised by and finally detached from the flavoprotein and cytochrome P450 respectively much more rapidly than the time it requires to traverse the minute distance between the two latter proteins. This concept is very similar to that of Green (1966) on the relative times involved in the transfer of reducing equivalents by cytochrome c from one complex of the mitochondrial electron transport chain to another.

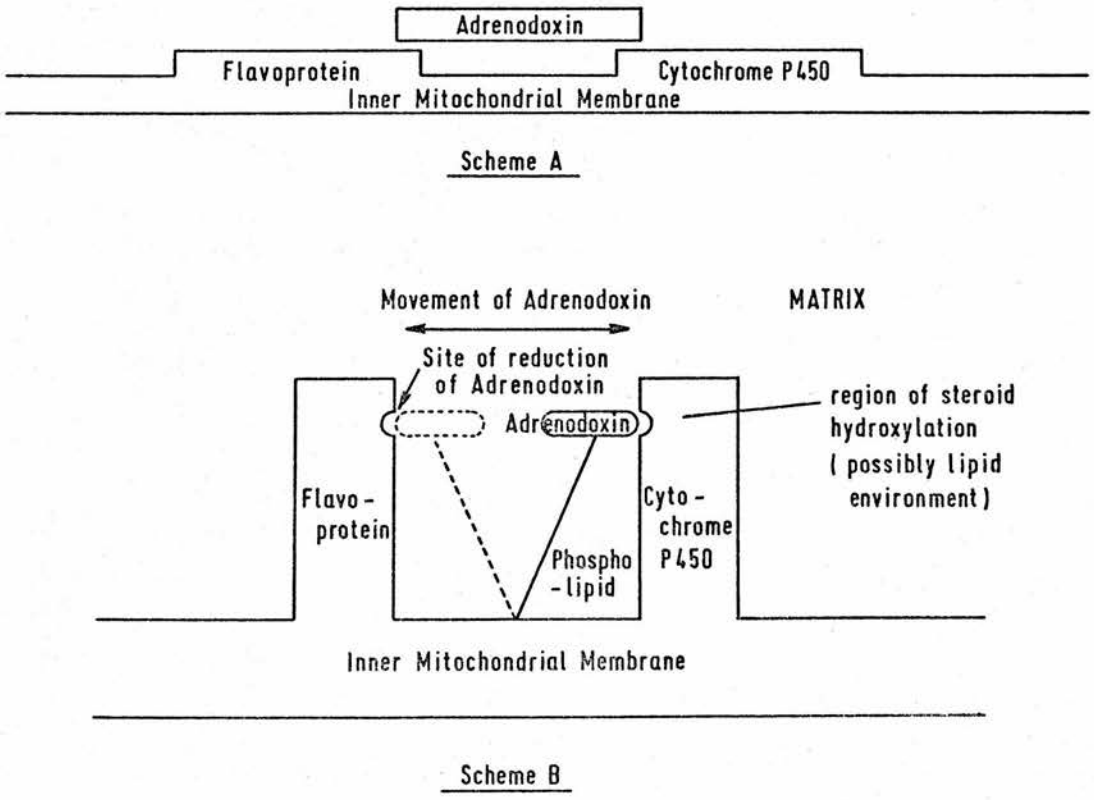


Fig. 59 Two schemes of possible interrelationships of components of adrenal cortex mitochondrial steroid hydroxylases

Table I showed comparisons of some characteristics of three general types of steroid hydroxylations in vertebrate tissues. It could be inferred from these characteristics that the iron-sulphur protein is a characteristic of the mitochondrial hydroxylases synthesising hormones and may have a regulatory function. One reason for the location of these hydroxylases within a mitochondrion and on the mitochondrial inner membrane (Satre, Vignais and Idelman, 1969; Yago and Ichii, 1969; Yago et al, 1970; cf. Billiar et al, 1971) could be to guard the delicate positioning of adrenodoxin relative to the other two proteins from streaming movements as are known to occur in the cytosol (Godina, Buffa and Barasa, 1961). Another possibility could be to exert a fine regulation on the composition of the immediate environment in which adrenodoxin functions and thus minimise fluctuations of corticosteroidogenesis due to temporary or local effects and those not indicative of the general metabolic state.

The importance of the composition of the immediate environment in which adrenodoxin functions as a means of regulation of its activity is seen by the results described in Chapter 8. Increasing the ionic strength of the assay medium leads to an initial enhancement of DOC 11β-hydroxylation (Fig. 45) up to a maximal rate of activity followed by an inhibition. The reduction of cytochrome c by NADPH in the presence of adrenodoxin and adrenodoxin reductase shows similar effects (Fig. 51). It has been noted that the hepatic 7α-hydroxylation of cholesterol, which is effected by cytochrome P450 and a cytochrome P450 reductase consisting of a flavoprotein only, is not very sensitive to ionic strength (Scholan, 1969). Similar fluctuations in the ionic strength do not appear to affect the activity of NADPH-cytochrome c reduction by hepatic microsomal cytochrome P450 reductase either (Ichikawa and Yamano, 1969). One difference between the adrenal

mitochondrial steroid hydroxylase and the hepatic microsomal steroid hydroxylase (Table I) is the presence of an iron-sulphur protein in the electron-transport system of the former and it could be that the effect of ions is exerted on the functioning of the iron-sulphur protein. This possibility is strengthened by the observation that the association of the adrenal iron-sulphur protein with the other two proteins is affected by ionic strength. There is an interesting parallel with the plant enzymes, ferredoxin and ferredoxin reductase, where it has been suggested that changes in salt concentration can regulate the photosynthetic electron transfer pathways (Nelson and Neumann, 1969b). Inhibition of some enzymic reactions by ionic strength is due to interference with protein-protein interaction as has been shown to account for the inhibition of the action of pepsin on bovine serum albumin (Cann, 1962; Cann and Klapper, 1961; Herriott, 1939; Loken et al., 1959) and of the reaction between cytochrome c and cytochrome c peroxidase (Mochan, 1970; Mochan and Nicholls, 1971; Nicholls and Mochan, 1971). Mochan (1970) considered that the interaction between cytochrome c and cytochrome c peroxidase was electrostatic as was the conclusion of Nelson and Neumann (1969b) with regard to the interaction between ferredoxin and ferredoxin reductase. The present observations on the effects of salts on the interactions of adrenodoxin with its reductase and cytochrome P450 lead to the conclusion that here too association is electrostatic. Edwards and Criddle (1966) and Williams and Thorp (1970) are among those who consider that the physiological binding of cytochrome c within mitochondria is due to both phospholipid-protein complexes and protein-protein complexes. It is considered that the same mechanisms may form the intramitochondrial associations of adrenodoxin.

In developing the concept of the "bioelectrode" or electrical half-cell constituting a specific part of an enzyme or enzymic biological unit, Hultin et al.(1969) referred to the possibility of some kind of "guidance" of ferricytochrome c molecules occurring rather

than to assume that random collisons account for the interaction of these electron-transporting molecules with beefheart cytochrome c reductase. It is considered that in the system under consideration in the present work similar (electrostatic) forces between adrenodoxin and the other two proteins and possibly the binding sites of protein and NADPH may be modified by the composition of the immediate environment.*

The effect of dipolar ions on *in vitro* steroid hydroxylations (Chapter 8) could indicate a method of regulation of corticosteroid biosynthesis in the mitochondrion *in vivo*. The several dipolar ions used and many structurally similar products are known to affect the dielectric constant of an aqueous solution (Cohn, 1936; Wyman, 1936). It is known that the dielectric constant of a solution can be affected by the presence of inorganic ions, proteins and dipolar ions which may have interacting influences (Cohn, 1936; Edsall and Wyman, 1935, 1958; Takashima, 1963). It is therefore proposed that the rate of intramitochondrial corticosteroid hydroxylations may be influenced by the dielectric constant of the immediate environment of the enzyme systems.

The observation that the relative inhibitions of steroid hydroxylation produced by salts of various monovalent ions could be related to the radius of their component anions and cations was interesting (Figs. 55 and 56). A similar effect was found by Imoto *et al.*(1969) when

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*Both adrenodoxin and cytochrome P450 are acidic proteins and Baxter (1959) and Lanyi and Stevenson (1970)discuss the possibility of an electrostatic "counter-ion charge shielding" effect of salts influencing the interaction between proteins containing excess acidic amino acids. This could be one of the mechanisms by which the interaction of adrenodoxin with cytochrome P450 is influenced but cannot account for the different effects of various monovalent anions and cations (Figs. 54, 55 and 56). "Chaotropic agents" such as perchlorate, thiocyanate and urea have also been found to affect the spectroscopic properties of ferredoxin and adrenodoxin (Cammack, Rao and Hall, 1971; Padmanabhan and Kimura, 1969; Petering and Palmer, 1970) but are not found in physiologically important amounts within the mitochondrion and are unlikely to be of metabolic significance.

investigating the activity of lysozyme which forms an enzyme-substrate complex. Although Imoto et al.(1969) attribute the effects observed to a fine conformational change at the active site, it could be that such a conformational change of the binding sites of adrenodoxin and NADPH or other factor which results in the experimental observations reported is caused fundamentally by the variation in dielectric constant with distance from a univalent ion. Fig. 60 shows this variation as calculated by various workers. Webb (1963) makes the point that the above treatment assumes that ions are point charges and takes no account of ionic radii, which will affect the final value of the dielectric constant. The fact that as regards their effects on steroid hydroxylation or lysozyme action the radii of both anions and cations have the same directional influence irrespective of sign of charge of the ion is significant since phenomena such as counter-ion charge shielding would not show this effect.

Numerous factors can affect the composition of the aqueous solution contained within the mitochondrion by alterations in the content either of solutes or of water and such changes can influence mitochondrial enzyme activity. It can be shown that the fluid surrounding the mitochondrion can influence the composition of its contents. The effect may be relatively direct such as the uptake by the mitochondria of a small inorganic ion added to the incubation fluid or indirect such as the accumulation of Ca^{++} being affected by inorganic phosphate concentrations (Rossi et al, 1966). Proteins are also known to influence these processes as for instance the effect of serum albumin on Ca^{++} uptake and swelling of intact mitochondria (Azzone, 1966). Chappell and Crofts (1966) found that isolated mitochondria behave as perfect osmometers, with a linear dependence of the mitochondrial water with external osmolarity. It is therefore likely

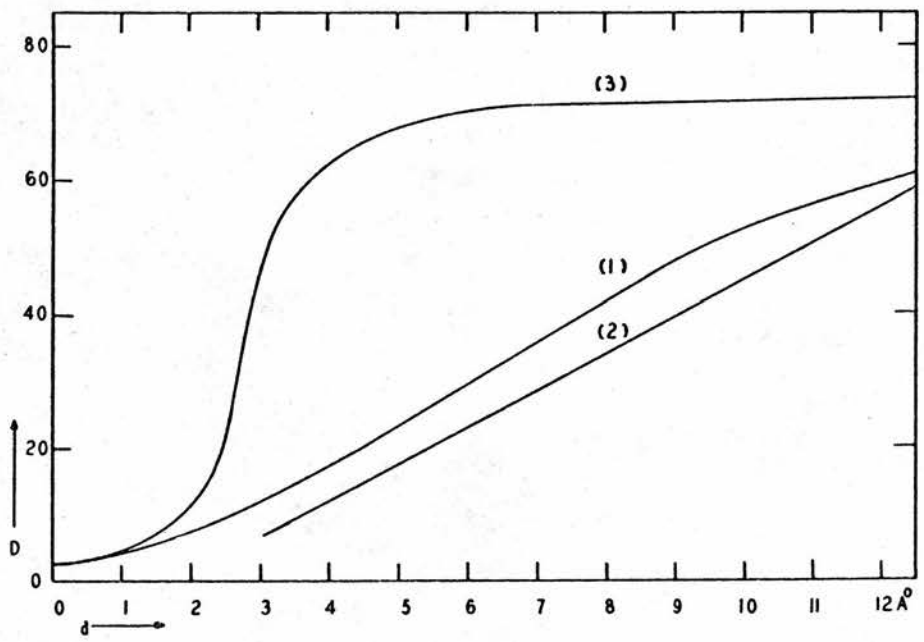

Fig. 60 Variation of the dielectric constant with the distance from a monovalent ion at 37.5°C as calculated by different methods (from Webb, 1963, p.246)

Curve (1) from Conway, Bockris and Ammar (1951)
Curve (2) from Schwarzenbach (1936)
Curve (3) from Grahame (1950) and Ritson and Hasted (1948)

that under normal physiological conditions the relative proportions and concentrations of the intramitochondrial solutes and water are influenced by the condition of the cytoplasm by which they are bathed.

The influence of extramitochondrial osmolarity on mitochondrial water and the observed effects of ions on protein-protein interaction (Figs. 53 and 54) and on steroid hydroxylations (Figs. 55 and 56) may be two factors of prime importance in the regulation of mineralocorticoid synthesis. Three key hydroxylation steps (i.e. cholesterol side-chain cleavage, the 11β-hydroxylation to corticosterone and the 18-hydroxylation to 18-hydroxycorticosterone) which lie on the proposed main biosynthetic pathway of aldosterone (see Fig. 61) are both intramitochondrial and adrenodoxin-dependent (Kimura, 1968; Nakamura, Otsuka and Tamaoki, 1966; Omura et al, 1965b) and thus possibly susceptible to the effects of fluctuations in the composition of the intramitochondrial contents on protein-protein interaction in the hydroxylase systems.

The rate of aldosterone secretion in vivo is influenced by a host of physiological factors including the osmotic pressure of the blood plasma (McLean, 1960), alteration of Na^+/K^+ ratio of the blood plasma (see Glaz and Vecsei, 1971; McKerns, 1969), bleeding, posture, Na^+ deprivation, K^+ administration and other factors which may be expected to affect the volume and osmotic concentration of the body fluids and electrolyte content and concentration of the plasma (Laragh and Kelly, 1964). Aldosterone biosynthesis in vitro is influenced by the osmotic pressure of the medium even at a normal Na^+/K^+ ratio (Purjesz et al, 1960), alteration of the Na^+/K^+ ratio even at a constant electrolyte concentration (see Glaz and Vecsei, 1971; McKerns, 1969), ACTH and Angiotensin II. However Baumber et al.(1971) have made in vivo studies with dogs and concluded that the effects on aldosterone production by

all four stimuli Angiotensin II, sodium depletion, hyperkalemia and ACTH are mediated by increases in the potassium content of the adrenal cortex cells with or without concomitant changes in sodium. (In this connection Chappell and Croft (1966) discuss the effects of Gramicidin A and Valinomycin on the permeability of the mitochondrial membrane to alkali metal ions.) Glaz and Vecsei (1971) have recently also suggested that there is good reason for believing that there exists a mechanism governing adrenal aldosterone production in addition to those already known.

These findings may be considered in relation to the evidence reported in the present work on the dissimilar effects of inorganic salts on the associations of the proteins transporting reducing equivalents for adrenal mitochondrial steroid hydroxylations and the relationship observed between the ionic radius and the effect of a specific monovalent ion on corticosteroid synthesis. While it is generally true that sodium and potassium ions (and NH_4^+) are the significant monovalent inorganic cations found in plasma, experiments with lithium, caesium and rubidium *in vivo* as well as *in vitro* demonstrate convincingly that lithium has an effect on aldosterone secretion similar to that of sodium while potassium and the other monovalent cations have the opposite effect (Bach *et al.*, 1960, 1961, 1967; Bartter *et al.*, 1964; Muller, 1965). The evidence that lithium and sodium ions have similar effects on aldosterone synthesis which are however opposite to those of monovalent ions of "large" radius (i.e. potassium, caesium, ammonium, rubidium) is significant. The hypothesis is advanced that within the adrenocortical mitochondrion the output of aldosterone is regulated by the balance of relative concentrations of monovalent inorganic ions of smaller radius such as sodium to those of larger radius such as potassium. Fluctuations to either side away

from the "normal" or "optimal" level whether caused by potassium loading or sodium deprivation or the effects of factors such as Angiotensin II or ACTH would affect corticosteroid hydroxylations and thus mineralocorticoid biosynthesis by affecting the rate at which reducing equivalents are transferred from the flavoprotein to cytochrome P450 by adrenodoxin. If the findings and conclusions of Baumber et al. (1971) are confirmed that ACTH is one of the agents causing a rise in adrenal cortex tissue potassium and thus providing an optimal "ionic environment" (Baumber et al, 1971) for steroidogenesis then the findings of Muller (1965, 1966, 1968) that all four agents which directly stimulate aldosterone production also enhance the conversion of cholesterol to pregnenolone are explicable by a common mechanism. Haning, Tait and Tait (1970) report similar effects on steroid output and corticosterone to aldosterone conversion by isolated adrenal cells which is added evidence that these mechanisms are operating at cellular levels.

The results seen in Fig. 55 on the relative inhibition of DOC 11β-hydroxylation by monovalent cations are however apparently anomalous in that monovalent cations of larger ionic radius give a greater inhibition of the conversion of deoxycorticosterone to corticosterone than do monovalent cations of smaller radius. However it has been long recognised (see Laragh and Kelly, 1964) that the physiological stimulus for increased aldosterone secretion is elevated potassium or reduced sodium levels. Since the pathway deoxycorticosterone $\longrightarrow$ corticosterone $\dashrightarrow$ aldosterone is commonly thought to be the main one of aldosterone biosynthesis of those possible (Fig. 61) the evidence that potassium apparently tends to have a greater tendency to inhibit one of the steps of this pathway would appear to conflict with other evidence. This however is probably explained by the fact that the

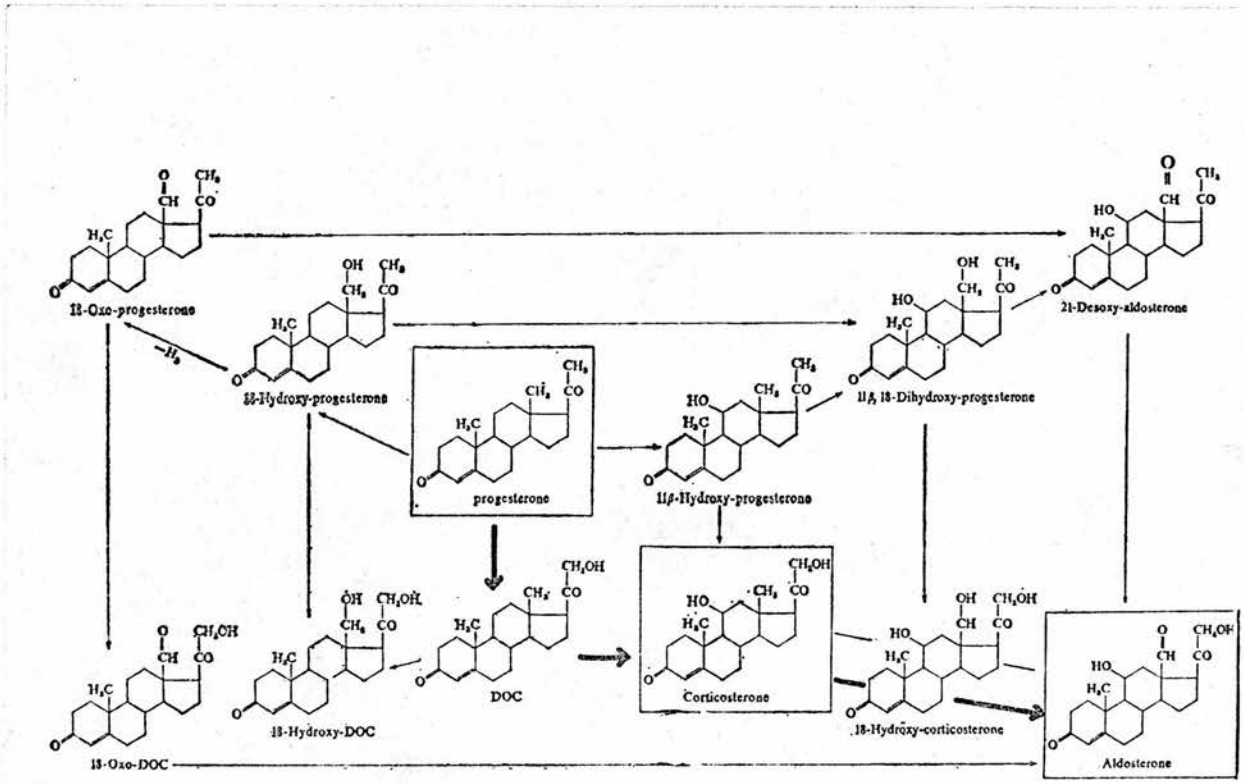

Fig. 61 Postulated pathways of aldosterone biosynthesis

(from Glaz and Vecsei, 1971)

The pathway depicted by heavy arrows is thought to be quantitatively the most important, under normal conditions.

initial conditions in which the assays (Figs. 55 and 56) were performed were chosen, especially as regards buffer concentrations, to give maximal hydroxylation activity. Further additions of salts in the form of halides of monovalent cations therefore led to inhibition of the rate of hydroxylation. However lower ionic concentrations within the mitochondria *in vivo* could lead to increased proportions of potassium ion resulting in an enhancement of mineralocorticoid synthesis (cf. Fig. 45 which shows the stimulating effect of initial increases of ionic strength) and sodium ion having the opposite effect.

In concluding the introductory chapter it was indicated that a principal objective of the present investigation was to examine the components of adrenal mitochondrial cytochrome P450 reductase with the aim of getting a better insight into the functioning of the steroid hydroxylating systems in adrenal mitochondria. It would appear likely from the results discussed above that adrenodoxin, the iron-sulphur protein component of the mitochondrial cytochrome P450 reductases, may be a regulatory particle the functioning of which is influenced by the composition of the intramitochondrial contents. It is likely that the amounts of inorganic ions, dipolar ions and water found within the mitochondrion of the adrenal cortical cell is influenced by the condition of the cell-sap which in turn is affected by that of the blood plasma. It has already been noted that the adrenal glands have a very active circulation (see Houssay and Molinelli, 1926). Thus changes in the composition of the blood plasma could relatively rapidly influence the composition of the adrenal intramitochondrial contents. Recently Hultin *et al.* (1969) referred to beefheart cytochrome c reductase as a "bioelectrode" or an electric half cell to and from which electrons can be transferred. By extension the complete unit depicted in Scheme B (Fig. 59) protruding into the intramitochondrial space may

be compared to the sensory mechanism of a self-regulating industrial chemostat or fermentation plant sampling the contents of the mitochondrial matrix and by means of pulses of signals (in this case electron transfer and hormone elaboration) initiating chains of regulatory devices designed to correct (in due course) any fluctuations which may occur in the composition of the matrix. The sensitivity of the electron-transferring mechanism to small imbalances in the intramitochondrial ionic environment would result in these fluctuations being "amplified" and signalled to effector organs such as the kidney by alterations in the production of the potent transmitters of information, the corticosteroid hormones. The action of the hormones on effector organs such as the kidney would result in early compensatory action to prevent gross imbalances which would disturb other metabolic systems as well. Chester Jones and Bellamy (1964) in their discussion of the homeostatic regulation of the vertebrate body with special reference to the functioning of the adrenal cortex observe that a single embryological tissue, the mesodermal blastema, gives rise to the renal, adrenocortical and gonadal structures. They observe (a) that functionally the adrenal cortex and kidney are closely associated, (b) that it is by the precise regulation of renal and extrarenal functions by adrenocorticosteroids that the water and electrolyte composition is maintained within a narrow range, and (c) that it is possible that the original and primary role of corticosteroids was and is in the control of water and electrolyte flux. Thus, assuming that the composition of the cellular intramitochondrial contents is related in some way to that of the body fluids, this system would be a typical closed-loop feedback mechanism composed of several transfer functions (Machin, 1964) and ensuring what Claude Bernard (1878) termed the stability of the internal environment and Cannon (1929) "physiological homeostasis".

APPENDIX A

ABBREVIATIONS AND DEFINITIONS

The following abbreviations have been used in the text:-

ISP	Iron-sulphur protein. A member of the class of proteins containing non-haem iron and acid-labile sulphur. By convention the term ISP includes the rubredoxins, which, although showing many of the characteristics of the class, do not however possess acid-labile sulphur.
PPNR	Photosynthetic pyridine nucleotide reductase
HiPIP	High potential iron protein
FP	Flavoprotein
Chol.	Cholesterol (Cholest-5-en-3β-ol)
Pregnenolone	3β-Hydroxy-pregn-5-en-20-one
Progesterone	Pregn-4-ene-3:20-dione
DOC	11-Deoxycorticosterone (21-Hydroxypregn-4-ene-3:20-dione)
Corticosterone	11β:21-Dihydroxy-pregn-4-ene-3:20-dione
Aldosterone	11β:21-Dihydroxy-3:20-dioxopregn-4-en-18-al (18 → 11 -hemiacetal)
FAD	Flavin -adenine dinucleotide
FMN	Flavin mononucleotide
NAD(H)	Nicotinamide-adenine dinucleotide (reduced)
NADP(H)	Nicotinamide-adenine dinucleotide phosphate (reduced)
3'-5' cyclic AMP	3'-5' cyclic Adenosine monophosphate
ATP	Adenosine 5'-triphosphate
G-6-P	D-Glucose-6-phosphate
G-6-P-D	D-Glucose-6-phosphate dehydrogenase
GSH	~~reduced~~ Glutathione
GSSG	Oxidised glutathione or glutathione disulphide
ACTH	Adrenocorticotrophic hormone (corticotropin)

DCPIP	2,6-Dichlorophenolindophenol
Bathophenanthroline sulphonate	4,7-Diphenyl-1,10-phenanthroline sulphonate
Tris	Tris (hydroxymethyl) aminomethane
DEAE-	Diethylaminoethyl-
CM-	Carboxymethyl-
TLC	Thin-layer chromatography
GLC	Gas-liquid chromatography
EPR	Electron paramagnetic resonance (electron spin resonance)

Steroid hydroxylations studied

The enzymatic steroid hydroxylations investigated experimentally in the course of this work were the side-chain cleavage of cholesterol to pregnenolone and the 11β-hydroxylation of 11-deoxycorticosterone to corticosterone.

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